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Epigenetic effects of ethanol on liver and gastrointestinal injury

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Abstract

Alcohol consumption causes cellular injury. Recent developments indicate that ethanol induces epigenetic alterations, particularly acetylation, methylation of histones, and hypo- and hypermethylation of DNA. This has opened up a new area of interest in ethanol research and is providing novel insight into actions of ethanol at the nucleosomal level in relation to gene expression and patho-physiological consequences. The epigenetic effects are mainly attributable to ethanol metabolic stress (Emess), generated by the oxidative and non-oxidative metabolism of ethanol, and dysregulation of methionine metabolism. Epigenetic changes are important in ethanol-induced hepatic steatosis, fibrosis, carcinoma and gastrointestinal injury. This editorial highlights these new advances and its future potential.

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Key words: Alcohol; Alcoholic liver disease; DNA methylation; Epigenetics; Ethanol; Gastrointestinal injury; Histone modifications; Liver injury

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INTRODUCTION

Ethanol actions are diverse and fascinatingly complex. Chronic ethanol causes injury to almost all organ systems including liver and gastrointestinal (GI)^[1] and has serious medical and public health implications^[2]. Alcohol increases the risk for hepatocellular carcinoma (HCC) and colon

cancer. Although these effects of ethanol are now widely known, our knowledge on the mechanisms of actions of ethanol at the subcellular and molecular levels is poor. Therapeutic tools to control or reverse the ethanol-induced cellular damages, such as alcoholic liver injury, are also lacking. In addition to its direct actions, ethanol-induced effects are also mediated by oxidative [e.g. acetaldehyde, reactive oxygen species (ROS)] and non-oxidative [e.g. phosphatidylethanol (PEth), fatty acid ethyl ester (FAEE)] metabolites/products and impairment in the methylation process. It is the combination of these metabolic stress pathways, termed as “ethanol metabolic stress” (Emess), which contributes to the epigenetic effects of ethanol (Figure 1).

The question of how a single cell can differentiate into many different cell types in a multicellular organism has long led to the hypothesis that additional information that regulates genomic functions must exist beyond the level of the genetic code. This concept led to the introduction of the term ‘epigenetics’ in the 1940’s, a term that has now evolved to mean heritable changes in gene expression that do not involve changes in DNA sequence^[3-5]. Interestingly, these epigenetic changes are heritable and normally stably maintained. They are also reversible. The molecular basis of epigenetics has largely focused on mechanisms such as DNA methylation and histone modification. In fact, emerging evidence indicates that both mechanisms act in concert to provide stable and heritable silencing.

ETHANOL EFFECTS ON DNA METHYLATION IN RELATION TO HEPATOCELLULAR AND GASTROINTESTINAL INJURY

DNA methylation specifically occurs at the C5 position of cytosine residues that are associated with CpG dinucleotides. Eighty percent of all CpG dinucleotides in the mammalian genome are methylated. The remaining unmethylated CpG residues are mostly located in the promoter regions of constitutively active genes and are referred to as CpG islands. Methylation of DNA is known to modulate transcriptional repression, genomic imprinting and modulation of chromatin structure^[4,5].

Global hypomethylation involves mainly repetitive sequences but hypomethylation of coding regions may also occur^[6]. Hypermethylation of normally unmethylated genes can result in silencing of tumor suppressor genes. Stepwise distinct methylation events are likely to be the features of the sequence from hepatitis to HCC and may

contribute to the process of hepatic carcinogenesis^[7,8]. Regional hypermethylation and global hypomethylation are also well recognized in gastrointestinal cancer^[9-12].

Only a few studies have addressed regional methylation of DNA in relation to alcohol and cancer. Alcohol either alone or in combination with tobacco has been shown to be an important risk factor for oral cancer^[13,14]. Promoter hypermethylation of p16INK4a, p14ARF, RB1, p21Waf1, p27Kip1, PTEN, p73, O⁶-methyl guanine DNA methyl transferase (O⁶-MGMT), and GST-P genes has been examined in relation to smoking and alcohol use. Overall, gene methylation can be detectable in 46.9% of samples and is closely correlated with tobacco use and/or alcohol consumption^[15]. The relative risk of alcohol consumption for the development of esophageal cancer is also very high^[16] and alcohol potentiates chemical carcinogenesis of the esophagus induced by nitrosomethylbenzylamine^[17]. Alcohol consumption has also been shown to be a risk factor for head and neck cancers that usually originates from the aerodigestive tract. Interestingly, p15 promoter hypermethylation has been observed in the healthy individuals who are smokers and/or alcohol consumers^[18], suggesting that hypermethylation plays a significant role in progression of cancer. Although alcohol consumption is not a significant risk factor for gastric carcinoma compared to oral or esophageal cancer, both smoking and alcohol consumption are associated with a higher risk of gastric cancer with hypermethylation of the hMLH1 gene promoter. Hypermethylation of the hMLH1 gene promoter is inversely correlated with mutation of the p53 gene^[19]. In a recent study, promoter hypermethylation of APC, p14 (ARF), p16 (INK4A), hMLH1, O⁶-MGMT, and RASSF1A was observed in colorectal cancer (CRC). For each of the tested genes, the prevalence of promoter hypermethylation is higher in CRCs derived from patients with low folate/high alcohol intake when compared with CRCs from patients with high folate/low alcohol intake^[20].

Although methylation changes have been described as stable for aging and carcinoma, recent studies have shown that epigenetic alterations are also dynamic as observed in inflammatory responses and tissue injury^[21]. Altered DNA methylation occurs after alcohol consumption during initial periods of alcohol abuse. Global hypomethylation of DNA in liver after long term ethanol exposure has been reported^[22] but hypermethylation of DNA from peripheral blood cells after ethanol consumption has also been reported in human subjects with alcohol dependence^[23]. Regional hypomethylation of the c-myc gene occurs in liver after long term consumption of alcohol^[22]. Another study showed that chronic alcohol consumption produces global genomic DNA hypomethylation in the colonic mucosa^[24].

Four known or putative mammalian DNA methyl transferases have been identified thus far: DNMT1, DNMT2, DNMT3a and DNMT3b. In contrast to types 1 and 3, the function of type 2 DNMT remains less clear. Mammalian DNMT2 does not methylate CGs. Non-CG cytosine methylation is also reported^[25]. Increased expression of DNA methyl transferases occurs in hepatocellular carcinoma^[26] and gastrointestinal cancer but this increased expression is associated with both

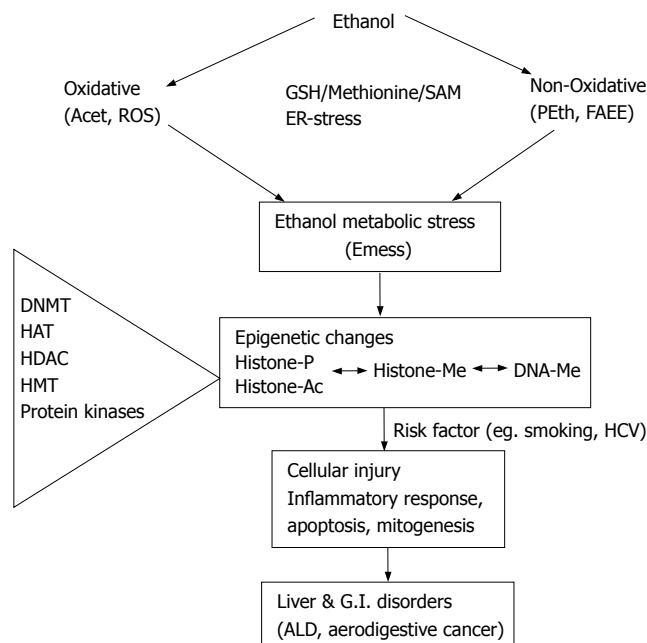


Figure 1 A diagram depicting relationship among ethanol metabolic stress, epigenetics and tissue injury. Acet: acetaldehyde; ALD: alcoholic liver disease; DNMT: DNA methyl transferases; ER: endoplasmic reticulum; FAEE: fatty acyl ethyl esters; GSH: glutathione; HAT: histone acetyl transferases; HCV: hepatitis C virus; HDAC: histone deacetylase; HMT: histone methyl transferases; PETh: phosphatidylethanol; ROS: reactive oxygen species; SAM: S-adenosylmethionine.

hypomethylation and hypermethylation of DNA^[27-29]. Decreased DNA methylation with a concomitant decrease in DNA methyl transferase activity after ethanol exposure of pregnant rats has been reported in fetal tissues^[30]. Decreased activity of methyl transferase has been reported in peripheral blood cells from alcoholics but with a concomitant increase in DNA methylation^[31]. This raises the possibility of regional methylations in a gene specific manner.

ETHANOL AND EPIGENETIC MODIFICATIONS IN HISTONE

Chromatin is the entire DNA-protein complex packaged into chromosomes. It exists as a highly ordered structure and is composed of repeated nucleosome subunits. Each nucleosome contains a core of histone around which DNA is wrapped. Eukaryotes have five major classes of histones: H1, H2A, H2B, H3, and H4. Histones were once thought as static, non-participating structural elements; and now considered integral and dynamic components in the machinery responsible for regulating gene transcription^[32]. The core histones (e.g. H3) have a similar structure with a basic N-terminal domain, a globular domain and a C-terminal tail. Modifications of histones can occur by mechanisms involving acetylation, phosphorylation, methylation, ubiquitination, sumoylation and ADP-ribosylation, *etc.* Some of these post-translational modifications affect packaging of genes, increase accessibility of transcription factors to DNA templates and initiate transcriptional processes^[32,33]. Such modifications can serve as 'co-activators' (e.g. acetylation,

methylation) or 'co-repressors' (e.g., deacetylation) or 'gene silencers' (e.g. methylation). In histone H3 from most species, the main acetylation sites include lysines 9, 14, 18 and 23. A steady state balance between two key enzymes, histone acetyl transferase (HAT) and histone deacetylase (HDAC), is crucial in this process. Various HATs^[34] and HDACs^[35] have been identified (about 15 types of HATs and 10 types of HDACs). H3 acetylation at lysine 9 or at lysine 14 plays a role in chromatin assembly^[32], gene expression^[36,37] and apoptosis^[38]. Histones, particularly H3 and H4, are methylated at a number of lysines (Lys) and arginine residues. The major sites of lysine-methylation identified are: Lys4, Lys9, Lys27, Lys36, and Lys79 on H3 and Lys20 on H4. In addition, lysine residues can be methylated in the form of mono-, di- or trimethylation and this differential methylation provides further functional diversity to each site of Lys methylation^[4,39]. Emerging evidence suggests that DNA and histone methylation likely have a cyclical and mutually reinforcing relationship, and both are required for stable and long-term epigenetic silencing^[4,39,40]. Lysine 9 is also interesting in that this site can be either methylated (gene silencing) or acetylated (gene activation). Histone H3 can also be phosphorylated at ser-10 and ser-28 by cellular protein kinases^[4,32,39]. The precise pattern of histone modification has been suggested to mediate biologically diverse effects and proposed as the 'histone code' hypothesis^[32]. The relationship among Emess, histone modification, DNA methylation and changes in the expression level of genes is an emerging topic of investigation.

HISTONE ACETYLATION BY ETHANOL IN LIVER

Initial studies with primary cultures of rat hepatocytes have established important characteristics of ethanol-induced histone acetylations. Ethanol causes a dose- and time-^[41] dependent selective acetylation of histone H3 at Lys9 (H3AcK9). Other H3 lysine residues i.e. Lys14, Lys18 and Lys23 are not acetylated under these conditions. Trichostatin A, a reversible HDAC inhibitor, shows an increase in H3 acetylation. These increases in acetylation are not due to the increased expression of H3 protein as their levels do not change. It is also not due to the simple physical effect of ethanol since it requires more than 4 h of ethanol exposure to elicit H3 acetylation. The acetylation is reversible when ethanol is withdrawn after 24 h of treatment.

Ethanol causes activation of p42/44 MAPK, p38 MAPK and JNK in hepatocytes, while inhibition of p42/44 MAPK and JNK results in inhibition of ethanol-induced acetylation^[42,43]. These results indicate that MAPK signaling plays a role in ethanol-induced epigenetic effects.

Ethanol acutely affects histone acetylation *in vivo*. Intragastric administration of ethanol increases 2-3 fold compared to the level of acetylated H3-Lys9 in the liver after 12 h, but has no effect on Lys14, Lys18 and Lys23. Further analysis indicates that the increased acetylation is tissue specific as it is noted in liver, lung and spleen but not in tissues from the brain, heart, kidney, muscle, vessels,

stomach and intestine. Thus ethanol-induced histone H3 acetylation appears to be organ specific^[44]. In rat liver stellate cells, ethanol increases H3 Lys 9 acetylation^[45] but its significance remains to be determined.

EFFECTS OF ETHANOL ON HISTONE METHYLATION

Ethanol also affects histone H3 methylations in an interesting manner. The influence of ethanol on histone H3 Lys9 and Lys4 methylations in primary cultures of rat hepatocytes is determined using site specific antibodies. Western blot analysis using methylated forms of Lys4 and Lys9 histone H3 antibodies can show dramatically opposing changes in the methylated forms. The Lys9 methylation decreases but Lys4 methylation increases in hepatocytes. These results indicate that, like H3 acetylation, histone methylation is also sensitive to ethanol. A longer incubation with ethanol for 72 h does not change this methylation, indicating that ethanol-induced methylation produces a longer effect than that observed for acetylation which declines after 24 h (Bhadra, U and Shukla SD, Unpublished). Thus modifications in H3 methylation are likely to be coupled to hyperacetylation and orchestrate the fine tuning of the chromatin status in hepatocytes exposed to ethanol.

ETHANOL-INDUCED HISTONE/CHROMATIN MODIFICATIONS AND TRANSCRIPTION

In hepatocytes exposed to ethanol, chromatin immunoprecipitation (CHIP) assays demonstrate the association of the acetylated H3-Lys9 with the alcohol dehydrogenase I (ADH 1) DNA domain in the nuclear chromatin^[43]. These data argue that ethanol-elicited epigenetic changes cause an increased association between acetylated H3 and specific genes, a process which favors transcription^[43]. It should be noted that circular dichroism spectrophotometry has shown altered chromatin confirmation in alcoholic rat liver, and this relaxed state of chromatin can promote transcription^[46]. Thus ethanol modulates histone/chromatin to influence transcriptional activation. Further relevance of such epigenetic changes to the expression of genes involved in ethanol-induced tissue injury therefore merits investigation.

RAS AND p53 AS MOLECULAR SWITCHES IN ETHANOL-INDUCED EPIGENETIC EFFECTS

Although structural alterations in genes contributing to HCC are evident in transformed hepatocytes, initiation of hepatocarcinogenesis takes place during the early stages of liver insult and is associated with epigenetic alterations^[7]. The progression of cell injury to carcinoma occurs due to triggering of 'some' molecular switches caused by a 'second hit', e.g. hepatitis C virus infection or other agents. Treatment of hepatocytes with ethanol causes apoptosis whereas alcohol enhances hepatic DNA synthesis in embryonic or transformed hepatocytes,

through potentiation of G-protein mediated ras/MAPK signaling. This underscores the importance of normal versus embryonic or transformed hepatocytes contributing to the opposing effects of ethanol^[47].

In this context, upregulation of ras signaling^[48,49] concomitant with down regulation of p53-dependent apoptotic pathway^[50-52] is seen in most cancers. Hypermethylation of apoptosis-related genes in ras transformed cells^[53] and hypermethylation of genes implicated in apoptosis in HCC associated with alcohol consumption, viral infection and aflatoxin contamination have been reported^[54]. Additionally, ras itself is subjected to epigenetic alteration by DNA methylation.

Hypomethylation of ras has been demonstrated in gastritis and gastric carcinoma^[49]. Ethanol induces ras activation in gastric epithelial cells^[55] and chronic alcoholic liver injury is associated with upregulation of ras activity^[56]. C-myc, which regulates both apoptosis and proliferation, is overexpressed in HCC and cooperates with ras in the development of carcinoma^[57]. Ethanol also causes an increased expression of c-myc, which is associated with hypomethylation of the c-myc gene^[22].

p53 is also a modulator of histone acetylation and methylation^[58,59]. Hyperacetylation of H3K9 with concomitant loss of dimethyl-H3K9 and increased methylation of H3K4 is seen with delayed suppression of hepatic alpha fetoprotein (AFP, a marker of embryonic phenotype) in p53-null mice^[60]. There is loss of p53 function by its hypermethylation in hepatocellular carcinoma^[61] and p53 mutation is common in gastrointestinal carcinoma^[62]. Apoptosis in chronic alcoholic liver injury is associated with p53 accumulation^[63]. In support of this, p53 null mice fed with ethanol exhibit suppression of apoptosis and increased proliferation of hepatocytes^[64]. The preceding observations strongly indicate that ras and p53 as switch targets play a role in ethanol-induced epigenetic mechanisms.

EMESS

Actions of ethanol are unique in that, ethanol or its metabolites have their own effects and can also sensitize (or desensitize) responses to other agonists. This "double edge" effect combined with the metabolic features of ethanol renders its actions multifaceted. Ethanol is oxidatively metabolized by alcohol dehydrogenase (ADH) or Cyt p450 to acetaldehyde which is next metabolized by aldehyde dehydrogenase (ALDH) to acetate^[65]. Phosphatidylethanol (PEth)^[66] and fatty acid ethyl esters (FAEE)^[67] are generated non-oxidatively from ethanol. Ethanol also causes generation of the reactive oxygen species (ROS) and modulates superoxide dismutases (SOD). Oxidative stress also leads to endoplasmic reticulum (ER) stress resulting in amplification of the injury^[68]. It is a combination of these metabolic stresses, including oxidative and non-oxidative, that causes injury to cells (Figure 1) and we term this as Emess.

A function of Emess is dysregulation of methionine metabolism leading to decreased generation of S-adenosylmethionine (SAM)^[22,69], a crucial methyl group donating step for DNA and histone methylations^[70]. Ethanol feeding affects several enzymes involved in

methionine metabolism including a decreased methionine synthetase activity and changes in hepatic SAM, S-adenosyl-L-homocysteine (SAH), SAM/SAH ratio^[70,71]. Dysregulation of methionine metabolism is further induced by folate deficiency associated with alcohol abuse^[72]. Disturbance in folate metabolism is also related to methylene tetrahydrofolate gene polymorphism^[69]. Another part of Emess is glutathione depletion^[72]. Glutathione depletion causes both global and regional hypomethylation of DNA^[73,74]. SAM administration decreases alcoholic liver injury when given for preventive intervention^[22,71]. Although SAM administration improves hepatic function, long term administration of SAM may have deleterious effects because of the accumulation of homocysteine. Betaine supplementation not only maintains SAM levels but also prevents homocysteine accumulation and elevates glutathione levels resulting in amelioration of ethanol-induced hepatic injury^[71]. Thus Emess-induced effects on glutathione and methionine levels have profound implications in epigenetic changes.

ACETALDEHYDE, DNA METHYLATION AND HISTONE ACETYLATION

One of the mechanisms underlying DNA hypomethylation is the direct inhibitory effects of acetaldehyde on enzymes implicated in DNA and histone methylations. Indeed acetaldehyde has been shown to inhibit both DNA methyltransferase^[30] and methionine synthase^[72,75].

Ethanol metabolism is involved in histone acetylation since inhibitors of alcohol dehydrogenase (4-methyl pyrazole) and aldehyde dehydrogenase (cyanamide) decrease ethanol-induced H3-Lys9 acetylation. This partial effect of inhibitors may imply that part of the ethanol effect on H3 acetylation, may also be independent of its metabolism. Since cyanamide increases the levels of acetaldehyde; and decreases acetylation of histones, acetaldehyde adduct formation is unlikely to account for the observed increases in H3-Lys9 acetylation. Interestingly, treatment of hepatocytes with ethanol metabolite acetate also elicits similar acetylation. Exposure of hepatocytes to acetaldehyde (0.01-1.0 mmol/L) for 24 h also increases H3AcK9. Antioxidant N-acetyl-L-cysteine (NAC, 10 mmol/L) decreases ethanol-induced H3 acetylation by about 50% in rat hepatocytes, suggesting that ROS may play a role in the acetylation^[41,43]. Ethanol thus causes characteristic changes in histone acetylation with sensitivity to ethanol metabolic/oxidative stress.

FUTURE ISSUES IN RESEARCH ON ETHANOL AND EPIGENETICS

Acute and chronic effects of ethanol on DNA methylation and regional hypermethylation or hypomethylation have yet to be established. Likewise, the effects of ethanol on promoter methylation of repetitive sequences as well as key genes that are implicated in survival and regeneration of liver remain to be explored. A comprehensive investigation into the molecular steps involved in ethanol-induced epigenetic changes and inter-relationships (cross-talks)

among epigenetic modifications, i.e. DNA methylations, histone methylations, is warranted. It will be interesting to examine the specificity of the effect of ethanol on individual DNA methyl transferases and histone methyl transferases. The effect of ethanol on histone acetyl transferases^[43] or on protein kinases involved in histone phosphorylation^[76] has to be ascertained. In parallel, the role of demethylases or deacetylases also needs to be assessed. In therapeutic strategies, drugs which modify the enzymes involved in these pathways can be predicted to alter ethanol-induced tissue damage and should constitute an important goal for future investigations. Additional measures, other than SAM or betaine, to suppress Emess and replenish hepatic glutathione by other agents (e.g. vitamin E, folic acid) should be considered. It must be mentioned here that ethanol-induced epigenetic changes are not limited to liver and GI. Evidence from other systems, e.g. fetal alcohol syndrome^[30], neuronal NMDA receptor^[77], synuclein^[78], brain^[79] and HERP gene^[80] further emphasizes the importance and potential role of epigenetic changes in alcohol-induced disorders in diverse systems.

Finally, it can be postulated that, as far as ethanol actions are concerned, the 'epigenetic' effects of ethanol may be more crucial than its effects on classical 'genetic alterations' like DNA deletions or mutations. This remains to be proven. Obviously, epigenetics is set to occupy the center stage of alcoholism research in the next decade.

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EDITORIAL

Screening in liver disease

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Abstract

A disease is suitable for screening if it is common, if the target population can be identified and reached and if both a good screening test and an effective therapy are available. Of the most common liver diseases only viral hepatitis and genetic hemochromatosis partially satisfy these conditions. Hepatitis C is common, the screening test is good and the therapy eliminates the virus in half of the cases, but problems arise in the definition of the target population. In fact generalized population screening is not endorsed by international guidelines, although some recommend screening immigrants from high prevalence countries. Opportunistic screening (case finding) of individuals with classic risk factors, such as transfusion before 1992 and drug addiction, is the most frequently used strategy, but there is disagreement whether prison inmates, individuals with a history of promiscuous or traumatic sex and health care workers should be screened. In a real practice setting the performance of opportunistic screening by general practitioners is low but can be ameliorated by training programs. Screening targeted to segments of the population or mass campaigns are expensive and therefore interventions should be aimed to improve opportunistic screening and the detection skills of general practitioners. Regarding genetic hemochromatosis there is insufficient evidence for population screening, but individual physicians can decide to screen racial groups with a high prevalence of the disease, such as people in early middle age and of northern European origin. In the other cases opportunistic screening of high risk individuals should be performed, with a high level of suspicion in case of unexplained liver disease, diabetes, juvenile arthropathy, sexual dysfunction and skin pigmentation.

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SCREENING STRATEGIES

Screening is defined as the application of a diagnostic test to an asymptomatic population in order to detect a disease at a stage when intervention may improve its outcome and natural history^[1]. For many years screening has attracted physicians and policy makers as a way of reducing the mortality of chronic illness with a cost effective model of medical care. It was perceived that generalized screening for the most common diseases, could reach the poorest population at higher health risk and in greatest need of medical care. With the advent of evidence based medicine, skepticism about the benefits of screening arose however in the medical community, particularly regarding the adverse consequences of false-positive and false-negative results of the tests utilized in the screening programs^[2]. It was evident that false positive results could produce anxiety, unnecessary examinations and incorrect labeling of part of the population, while false negative results could impair the cost efficacy of the screening. Criteria were therefore established to appraise the effectiveness and appropriateness of these programs^[3] and for their acceptability proof was required of evidence for the reduction of disease specific mortality from randomized controlled trials.

Together with the demand for more evidence based policies came also a better understanding of the different screening strategies. Screening can be performed in different ways and with different target populations.

(1) Population screening is the application of a test to the entire population, generally with age or sex, restrictions, e.g. mammography applied to women of more than 50 years of age.

(2) Targeted screening is the approach of screening only patients at higher risk for a specific disease, e.g. searching for atrial fibrillation in patients with a history of myocardial infarction, angina, diabetes mellitus, hypertension *etc*^[3,4]. Targeted screening utilizes existent disease registers in general practice and local health districts or prescribing information from computerized records.

(3) Opportunistic screening, also termed opportunistic case finding or simply case finding, looks for additional illnesses in a population already complaining of medical problems. In this strategy health professionals, most commonly general practitioners, apply a screening test

Table 1 Characteristics of the most common liver diseases and feasibility of screening

	Hepatitis C	Hepatitis B	Fatty liver	Hemochromatosis
Disease common and causing morbidity/mortality	Yes	Yes	Common, but increased mortality only if advanced NASH	Common, but only 1% of screened population with complications ^[13]
Target population identifiable	High risk groups	High risk groups	Obese, diabetics	Northern European ancestry
Screening test	HCV Antibody test	HBsAg test	Ultrasound	Transferrin saturation or genetic testing (unsettled)
Performance of test	Good	Good	Low PPV and NPV for fibrosis	Under investigation
Effective therapy	50% cure ^[14]	4%-24% HBsAg loss, 70% no progression ^[15,16]	Only lifestyle modification	Yes, but may be unnecessary

to individuals with specific risk factors for the disease and attending their offices with other medical problems. Opportunistic screening is the simplest and less expensive form of screening because there is no need of additional staff and of complicated reach out or recall procedures. The screening test is requested during the patient's consultation and the next appointment is scheduled immediately at the time of the first visit. This strategy however may miss a significant proportion of people who do not present for consultation, but who would otherwise benefit from treatment. Another drawback is that it can be conducted only in those countries with a well established primary care network.

(4) Surveillance is the application of targeted screening over time to a particular category at risk and already harboring a disease. Well known examples of this strategy are the use of periodic ultrasonography and upper gastrointestinal endoscopy in cirrhotic patients in order to detect early stage hepatocellular carcinoma or varices at higher bleeding risk. Another type of surveillance that has been proposed in the field of hepatology is the use of periodic liver chemistry for the detection of drug induced liver toxicity. The terms screening, case finding and surveillance are not uniformly used in the literature and are often inappropriately used interchangeably^[5]. In fact surveillance, strictly speaking is different from screening, being applied not to healthy and asymptomatic people, but to individuals who have already been recognized to suffer from a specific disease.

FEASIBILITY OF SCREENING IN LIVER DISEASE

The implementation of a screening program is requested to meet some basic criteria^[6]: (1) the disease must be common and produce significant morbidity and mortality. (2) The target population must be easily identifiable. (3) The screening test must have good sensitivity and specificity. (4) There must be well defined recall procedures (5) The test should be accepted by the population to be screened. (6) There must be an effective therapy for the disease.

Liver disease seems to meet at best only the first and third of the 6 established criterions. The three main causes of liver disease are viral hepatitis, alcoholic and non-alcoholic fatty liver, which are all quite common. Hepatitis C virus affects 200 million people in the world with an

expected increased mortality for end stage liver disease and hepatocarcinoma in the next 10-20 years^[7,8]. The global disease burden of Hepatitis B is also substantial: it is estimated that there are in the world 350 million chronic hepatitis B carriers^[9] and that 500 000 to 1.2 million people will die annually from the complications of HBV infection^[10]. The other most common causes of liver disease are fatty liver and alcoholic liver disease, which are widespread in the western world and a rising problem also in the developing world^[11]. However the natural history of fatty liver is less well defined compared to viral hepatitis and is characterized by concomitant cardiovascular risk factors such as the metabolic syndrome, diabetes and obesity. It is therefore difficult to predict its liver related mortality in the next 20 years. Alcoholic liver disease is, on the contrary, a multifaceted problem which is beyond the goals of population screening, even if physicians are using a wide array of diagnostic tools to detect alcohol problems on an individual basis. In addition to viral hepatitis, genetic hemochromatosis has also been proposed for population screening, being a common and treatable disease, but it is doubtful which is the optimal test to be used and if only people with northern European ancestry should be subjected to screening^[12]. The specific liver diseases potentially amenable to population screening are listed in Table 1.

In conclusion among the most common liver diseases only fatty liver lacks the characteristics that would render it feasible for a screening program. Both viral hepatitis and hemochromatosis can be considered for some form of screening and will be discussed in detail.

HEPATITIS C

Hepatitis C has many characteristics that render it potentially suitable for screening. In fact, it causes significant morbidity, high risk groups are identifiable as a target population and the screening test is good. The testing strategy utilizes the antibody test which has excellent specificity and sensitivity, if we exclude some false negative cases in acute infections and in the immunocompromised state^[17]. In these situations the diagnosis can be made with HCV-RNA by using amplification techniques. For the majority of patients the usual approach is to test them initially for the antibody and then to use HCV RNA to detect viremia and decide whom to treat^[17]. Current treatment of Hepatitis C is not entirely

Table 2 Indications for screening with anti-HCV antibody

Risk factors for which testing is indicated by all the guidelines	Risk factors for which testing is indicated by some of the guidelines	Additional risk factors for which testing is not formally recommended
Intravenous drug use (past and present)	Populations with high HCV prevalence ^[12,30]	Injections with reusable glass syringes ^[32]
Blood transfusion or transplantation before 1992 (or by known HCV positive donor)	Incarceration ^[12,33]	Heavy marijuana use ^[34]
Administration of clotting factors before 1997	Hepatitis B virus infection ^[12,33]	Promiscuous sex ^[34]
Clinical or biochemical evidence for chronic liver disease	Sharing intranasal cocaine equipment ^[12,33]	Poverty ^[34,35]
Percutaneous exposures to HCV	History of sexually transmitted disease ^[12,33] with genital erosions ^[28]	History of invasive procedures ^[36]
Haemophilia	Traumatic sex or vaginal sex during menstruation ^[14,33]	History of surgery ^[36]
Children born to HCV + ve mothers	Health Care Workers performing procedures at risk of transmission to the patient ^[37]	Beauty treatments ^[38]
HIV positivity		
Stable sexual partners of HCV + patients		

satisfactory because it can eradicate the virus in only half of the cases^[18,19], but in the future new treatments will be available with far greater efficacy^[14]. The low yield of treatment has prompted the US Preventive Services Task Force to dismiss Hepatitis C as a potential candidate for screening^[20]. Sufficient evidence from the literature has not found that screening and treatment of hepatitis C could prevent chronic liver disease and decrease mortality. This opinion has been challenged by other experts^[21] who pointed out that the lack of studies showing a favorable impact of antiviral therapy on mortality is due to the long history of the disease. According to these experts eradication of the virus with normalization of liver enzymes could be considered a surrogate marker for increased life expectancy, as indicated by studies with a long follow up of sustained responders to antiviral therapy^[22-24].

For this reason several international societies have now endorsed screening strategies for hepatitis C^[25-30]. Their official guidelines recommend selective screening targeted for high risk groups, but none of them endorse generalized population screening, mainly for the relatively low prevalence of hepatitis C outside the categories at risk. Other arguments against population screening are that nearly half of the detected patients will have normal ALT and a slow progressing disease^[31], that many patients will harbor conditions contraindicating antiviral treatment and last but not least, that many persons with mild disease will have deterioration in their quality of life after knowing the result of the test^[28]. These drawbacks limit the effectiveness of a mass screening program and render it not cost effective. The optimal method to detect HCV infection, according to all international guidelines is therefore to screen individuals with identifiable risk factors (Table 2).

There is general agreement among all the guidelines that there are major risk factors for hepatitis C and that all individuals with one or more of these factors should be screened with the antibody test. These factors are listed in the first column of Table 2 and basically include intravenous drug use and blood transfusions before 1992, the year when HCV testing of blood units was introduced. Other categories at increased risk that should be tested are

the hemophiliacs, HIV positive patients and children born to HIV positive mothers. Although many studies have shown that HCV transmission is rare^[39-41] or virtually non-existent^[42] in stable monogamous couples, all the guidelines recommend testing of the spouse or partner, mainly for reassurance.

Though there is consensus on the most important risk factors some disagreement exists for other conditions at risk, which are mentioned only by some of the guidelines and not by others. For example the Canadian College of Family Physicians emphasizes the problem of sexual exposure and recommends testing in case of promiscuous, traumatic or vaginal sex during menstruations^[33]. The Italian guidelines suggest testing individuals with high risk sexual behavior only if they have a history of genital erosions^[28]. Promiscuous sex is not considered an indication for testing by the other guidelines, being discussed in detail only by the US Preventive Services Task Force who examining 4 large population studies^[43-46] could not rule out a concealed association between promiscuous sex and unacknowledged drug use^[20]. For this reason systematic testing of individuals with multiple sex partners or homosexuals was not recommended. Another debated issue is whether health care workers performing exposure prone procedures should be screened for hepatitis C. For exposure prone procedures it is indicated that the performance of invasive procedures are putting the patient at risk to contract a blood borne virus. From 1994 to 2002 fifteen British patients have been infected by health care workers (HCWs) carrying the hepatitis C virus. Following these incidents the British Department of Health recommended screening all HCW performing or intending to perform exposure prone procedures first with an anti HCV antibody test and if they test positive, with an HCV RNA assay. Anti-HCV positive HCWs carrying the virus are restricted from performing such procedures until they receive treatment and get rid of the virus^[37]. This opinion is not shared by the other guidelines that do not recommend HCW screening, regardless of their type of duty.

Another source of disagreement is population screening. In spite of an apparent unanimity among the

guidelines against generalized population screening, two of them^[12,30] suggest to screen individuals coming from high prevalence countries, which is an indirect form of population screening. The two guidelines do not set a threshold to define a high prevalence country, but many experts think of Egypt and Pakistan, which have an anti HCV prevalence of more than 10% in the general population^[47-49]. Another particular form of screening a segment of the population is the testing of prison inmates, recommended by the Canadian Guidelines^[14,33].

Many other risk factors have been found to be associated with an increased risk of HCV infection in epidemiological studies, but none has proven useful in clinical practice. For example in the Mediterranean area many infections have been transmitted in the past through the use of reusable glass syringes. However, due to their widespread use, a positive history for this risk factor has a very low positive predictive value and is practically useless in the clinical setting^[32]. The same applies for the history of surgery and invasive procedures after a French study demonstrating that screening for these additional risk factors is not cost effective in general practice^[50].

In conclusion, there is only partial consensus among the international guidelines on the appropriate risk factors to be used in the clinical setting, but there are other problems to be considered. First, none of the guidelines indicate how the screening strategy should be actuated-e.g. if the screening should be targeted or opportunistic. In other words it is not clear if an active search for individuals belonging to categories at risk should be implemented or if the less demanding opportunistic screening will suffice. It is true that hemophiliacs, hemodialysis patients, HIV positive individuals and drug addicts on detoxification programs are usually registered on files and record charts that could be accessed for screening. In this case, individuals at risk easily recalled, but what about people transfused earlier than 1992? Many of them are women who received few blood units after delivery, a bad habit of the past, but are not aware of being at risk. Should we actively search for them in the blood bank files of our hospitals or simply wait until they come to their general practitioner and rely solely on his history skills? In addition the patient's privacy could be at issue when browsing into these records. There are also doubts that an organized, widespread targeted screening would be cost effective, as shown in a study conducted in a pilot French area highlighting the difficulty and cost of mobilizing a considerable number of health professionals in the screening campaign^[47]. In the only cost effectiveness study published in the literature, Josset^[36] calculated a cost of 2247-3318 € per identified case for the reference strategy, consisting of the opportunistic screening of individuals at risk. This cost skyrocketed to 10994 € for targeted organized screening with mobilization of health professionals^[47], which is surely a waste of money considering that only half of the detected cases can be cured.

If we admit that the most rational approach is opportunistic screening and not population or targeted screening, we are faced with the problem of assessing the performance of general practitioners in detecting the patients at risk. Many studies have addressed this issue,

chiefly using questionnaires, mail surveys and phone interviews, but the overall results are confusing. Some of them found a good knowledge of risk factors by family physicians^[51-54], but others have not^[55-60] irrespective of the geographical areas. The actual behavior of family physicians in real practice was investigated in one study, by scrutinizing the medical records of 229 hepatitis C patients from 26 primary care clinics in Michigan^[61]. It was found that testing was initiated based on physician-identified risk factors only in 20% of the patients, while the majority of them were tested because of liver enzyme elevations, history of hepatitis B or by direct patient request. It is noteworthy also that a minority of the patients already knew their HCV status and that only 10% of them were asked about risk factors during their first visit at the clinic. This means that, in a real practice setting, family physicians are less aware of the risk factors of hepatitis C, than when questioned in formal interviews. In another real practice study comparing different screening strategies, HCV infection was found in only 5% of the tested patients^[62] showing that, irrespective of the adopted strategy, the yield of this screening is rather low. These real practice studies depict a bleaker scenario than what was apparent from questionnaire and interview studies. Hopefully the performance of family physicians can be improved by training interventions and by the participation of these physicians to hepatitis C networks, as shown by the French experience^[63]. Both the screening efficacy and the appropriateness of referrals can be ameliorated by long term training and by the adoption of specific guidelines for family practice. These guidelines consist of easy to use flow charts for the identification and referral of the patients and can be utilized by general practitioners in their offices^[64]. The same goals can be achieved in far to reach rural areas by the use of CD based software, mailed to physicians practicing in the same areas. This comprehensive software contains the various aspects of the care of hepatitis C, including diagnosis, counseling, treatment and follow up^[65].

In summary, we can conclude that opportunistic screening of categories at risk is the best form of screening recommended in the literature, but that no consensus exists on some of the risk factors. Population screening is recommended only for some particular ethnic groups and only by some of the guidelines. In a real practice setting the performance of this type of screening by general practitioners is low but could be ameliorated by training interventions. The cost of other forms of screening is too high to merit consideration and therefore interventions should be aimed to improve the detection skills of general practitioners.

HEPATITIS B

The case of hepatitis B is completely different from hepatitis C. In fact, the treatment can eradicate the virus in only a small minority of the patients^[15,16,66], but an effective vaccine is available to prevent transmission of the infection. Screening therefore has been implemented in the categories at risk to optimize the vaccination strategies and not to detect infected individuals to be cured, as

in the case of hepatitis C^[67-69]. In some high prevalence populations such as Asian and Pacific island immigrants in the United States, some screening for treatment programs have been conducted. In the New York City program^[70] 1836 people were tested for HBV infection: 24% of them were found HBsAg positive and 90% returned for further evaluation and possible treatment. A total of 505 (27.5%) were negative for hepatitis B markers and were vaccinated with a discrete coverage rate (70% after the third dose). The results of the New York City program show that screening a high prevalence segment of the population is feasible and can identify in a cost effective way both susceptible people to be vaccinated and infected individuals to be treated. Control of hepatitis B is however achieved worldwide by means of vaccination and in fact the World Health Organization recommends the addition of HBV vaccine to all national immunization programs^[71]. The vaccination strategy depends on the prevalence of HBsAg carriers in the general population: in countries with intermediate and high prevalence (more than 2% and 8% of the general population), universal neonatal or infant vaccination is recommended. Even in countries at low risk (1%-2% prevalence) universal infant or adolescent vaccination is cost effective if the vaccine can be delivered efficiently^[67]. Only in countries at very low risk (around 0.05%) such as the United Kingdom, Scandinavia and the Netherlands a selective risk group vaccination could be more cost effective than universal vaccination^[67,72]. Regarding the categories at risk that should be screened and vaccinated, there is full consensus in the literature on their definition^[69,73]. These categories are listed in Table 3. Individuals with these risk factors should be screened with a case finding strategy in the context of vaccination delivery programs^[72] and those found to be HBsAg carriers evaluated by the specialist for possible treatment.

HEMOCHROMATOSIS

Hereditary Hemochromatosis is a genetic disorder of iron metabolism in which iron is excessively absorbed by the intestinal tract and accumulates in the liver, heart, joints, pancreas and other endocrine glands. There are 4 different forms of hereditary hemochromatosis: (1) HFE gene mutations which result in upregulation of iron absorption by intestinal cells (2) Juvenile hereditary hemochromatosis with mutations in the hemojuvelin gene HJV (type 2A) or in the HAMP gene (type 2B), (3) TFR-2 related hemochromatosis in which mutations in the transferrin receptor may lead to an increased uptake of iron by hepatocytes and (4) Ferroportin related iron overload in which mutations of the ferroportin gene cause the absence of ferroportin activity and the inappropriate iron sequestration within the reticuloendothelial cells^[74].

The most common form of hereditary hemochromatosis is related to the substitution of tyrosine for cysteine at position 282 of the HFE protein (C282Y): homozygosis for the C282Y mutation predisposes to iron accumulation and is found in 50%-95% of classic hemochromatosis, depending on the geographic areas. The highest frequency of this mutation is observed in people of northern European descent (0.3%-0.5% of the general population) while lower

Table 3 Categories at risk for hepatitis B that should be screened and vaccinated

Immigrants from high prevalence areas (> 8% population) Asia, Pacific Islands, Alaska, Greenland, Africa, Middle East, former USSR, Eastern Europe (except Hungary), Malta, Amazonian areas of Peru, Brazil, Bolivia and Venezuela
Refugees, adopted children, residents for more than 6 mo in the same areas
Blood transfusion before 1973
Drug addicts
Individuals with clinical or biochemical evidence for chronic liver disease
Percutaneous exposures to HBV
Haemophilia
Pregnant women
Haemodialysis
Household, sexual and needle sharing contacts of HBsAg patients
Sexual partners of HBsAg patients
Health Care Workers

frequencies (less than 0.1%) are found in Asians, Blacks and Hispanics^[75]. The second most common mutation of the HFE gene, linked to hemochromatosis, is the H63D mutation. One to 2 percent of people with compound heterozygosity for C282Y and H63D will eventually express the disease, while homozygosis for H63D does not seem to be associated with significant iron accumulation^[74]. A third mutation of the HFE gene, S65C has been described, but it is debated whether this mutation is associated with hemochromatosis in the compound heterozygous form with C282Y^[76]. Mutations in other genes different from HFE have been reported in Italian families with iron overload comparable to the classic HFE form^[77-79].

Hemochromatosis is a common disease and also amenable to treatment because venesection is safe and can change the natural history of the disease^[80,81]. It would therefore be advantageous to submit individuals to a screening test for hemochromatosis in order to detect the disease at an early stage, before the occurrence of damage to vital organs. Genetic testing of large samples of the general population in the USA (HEIRS Study: Hemochromatosis and Iron Overload Screening in a racially diverse population) and Norway^[75,82] showed that hemochromatosis is a common disease in non-Hispanic whites (0.44%) and Norwegian males (0.66%) while individuals of other racial origin show a much lower prevalence. For example only 0.027 of Hispanics and 0.014 of American Blacks were homozygous for the C282Y mutation. Asians have the lowest prevalence (0.00039%) of HFE mutations, but surprisingly have more iron overload than the other racial groups. Iron overload was defined as a serum ferritin greater than 300 mg/L in man and 200 mg/L in women plus serum transferrin saturation greater than 50% in men and 45% in women. These data clearly show that genetic testing is not sensitive enough to detect all cases of iron overload and neither is highly specific, since as reported in the HEIRS study 88% of men and only 57% of women homozygous for the C282Y mutation had elevated serum ferritin. In addition, it is by no means certain if these patients will develop significant life threatening complications over time. In fact in a large population based study

the prevalence of these complications in C282Y homozygosity was found as low as 1%^[13]. Other studies searching for a genotype-phenotype correlation in C282Y homozygosity have yielded varying results: increased ferritin levels were found in 19%-75% of these subjects with a median of 65%^[83,84]. Regarding liver damage approximately 50% of the C282Y homozygous with abnormal iron indexes in the Norwegian study underwent liver biopsy, and only a small minority was found to have cirrhosis^[82] indicating that finding a high ferritin and transferrin saturation does not necessarily indicate the presence of severe liver disease. The other genotypes linked with the development of familial hemochromatosis, such as the C282Y/H63D compound heterozygous, have a lower risk of iron accumulation^[85] and are not worth screening. Similarly the non-HFE related hemochromatosis, such as ferroportin disease and mutations in the HJV and TFRF-genes are much less frequent than the homozygous C282Y form and their diagnostic tests have not yet been validated for population screening^[86]. In conclusion genotypic screening is not the ideal screening tool for hereditary hemochromatosis because it is uncertain whether the detected individuals will progress into overt disease.

Many guidelines have addressed the issue of population screening in genetic hemochromatosis^[87,88], the most recent of which was published in 2005 on behalf of the American College of Physicians^[89]. In none of these guidelines is widespread population screening recommended, but the American College of Physicians leave the specialist and general practitioner free to perform a once in a life phenotypic screening of asymptomatic non-Hispanic white men with ferritin and transferrin saturation (phenotypic test). The central issue is the sensitivity and specificity of these tests to detect true hemochromatosis and which is the best cut off to be used. Schmitt and colleagues^[90] require that the gold standard be an independent demonstration of iron overload through iron deposition by liver biopsy or the amount of iron removed by phlebotomy. Among the main studies of the literature^[80,82,85], however, a small minority of the identified patients was further investigated with liver biopsy or the amount of depleted iron precisely calculated. Hemochromatosis was diagnosed in all of these cases and therefore phenotype testing seems to give a higher yield compared to HFE genotyping in detecting both iron overload and liver disease^[89]. The key point is that genotypic testing identifies many patients that will not progress into overt disease, while phenotypic testing circumvents this problem, because the combined use of serum ferritin and transferrin saturation is directly related to iron overload. Serum ferritin is unreliable as a sole marker, but is beneficial in classifying patients with regard to the presence or absence of cirrhosis^[88]. Serum ferritin has a high sensitivity but poor specificity for iron overload^[87] and therefore needs to be supplemented by the use of transferrin saturation. The best thresholds for these tests have been defined in the HEIRS studies and have already been discussed^[75]. Transferrin saturation has the advantage that it is independent of body mass index^[91] but care should be taken to draw the blood sample in the morning to avoid interference by the post absorptive state

and circadian rhythm^[88]. Another advantage of phenotypic compared to genotypic testing could be the negative psychosocial consequences derived by stigmatization of the patients by a positive genotypic test, albeit two studies addressing this problem did not provide evidence of negative psychosocial consequences to the patients^[92,93]. What can be said in summary regarding screening of this disease?

(a) Both the American Association for the Study of the Liver and the American College of Physicians deem that there is insufficient evidence for population screening, but recommend targeted screening of individuals at high risk. The target population should be: (1) patients with unexplained liver disease or known liver disease with elevated serum iron markers, (2) type 2 diabetics, (3) first degree relatives of hemochromatosis patients, (4) patients with early onset atypical arthropathy, cardiac disease or male sexual dysfunction, (5) unexplained changes in skin pigmentation. There is no data available to risk-stratify the patients according to these conditions and to recommend more intensive screening for a particular group. (b) Individual physicians can decide to screen persons belonging to racial groups with high prevalence of genetic hemochromatosis, such as people of northern European origin. This disease could be a candidate for opportunistic screening as part of routine health maintenance when Caucasian patients in early middle age are seen by general practitioners in their offices. This strategy however has not yet been validated by a cost efficacy study. (c) Screening should be performed once in life with serum ferritin and transferrin saturation using the thresholds defined by the HEIRS study.

OTHER GENETIC LIVER DISEASES

Alpha-1-antitrypsin deficiency and Wilson's disease are the other most important metabolic diseases that could potentially be considered for population screening. Alpha-1-antitrypsin deficiency is a common autosomal recessive condition which may cause liver cirrhosis in children and young adults. The classic homozygous form of alpha-1-antitrypsin deficiency affects 1:1800 newborns and predisposes to the early development of pulmonary emphysema, cirrhosis and hepatocellular carcinoma^[94]. Population studies conducted in Sweden have shown a wide variation in the expression of liver disease among the homozygous carriers of the z allele, which is associated with alpha-1-antitrypsin deficiency^[95,96]. In one of these studies only 10% out of 127 homozygotes followed for over 20 years were found to develop significant liver disease. In another more recent study conducted in Austria 16410 asymptomatic individuals were tested for the alpha-1-antitrypsin phenotype. Eighty six percent of them were found to be carriers of the normal Pi-MM phenotype, 7% were heterozygous for the Pi-Z phenotype, 5% heterozygous for the less common Pi-S phenotype and 0.7% were found homozygous for the Pi-ZZ phenotype. There was no difference both in liver related mortality and cumulative survival in the four groups of individuals, even after a very long period of follow up, that is after a median of 53 years^[97]. All these data show that other

genetic and environmental factors may modify the expression of this abnormal genotype and render alpha-1-antitrypsin deficiency unfeasible for population screening in newborns, children and young adults. It should also be stressed that alpha-1-antitrypsin replacement therapy is not effective in preventing liver disease and therefore the identification of affected individuals could only be used for the prevention of pulmonary emphysema^[98].

Wilson disease, caused by copper accumulation within the liver parenchyma, is another important genetic disease that could benefit from effective chelation therapy^[99]. In the early nineties a putative gene causing Wilson disease was discovered (ATP 7B) and it was named the WND gene. This offered promise for its use as a genetic marker of the disease and also for population screening^[100,101]. However, the mutations of the ATP 7B gene are quite complex and a recent study failed to show significant correlations between symptoms, ceruloplasmin levels, hepatic copper content and any specific pattern of mutations^[102]. The prevalence of Wilson's disease in the general population is also relatively low, being on average 30 affected individuals per million people^[103] and therefore screening for this disease on a widespread scale is not justified.

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Mutation screening of mismatch repair gene Mlh3 in familial esophageal cancer

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tions of Mlh3 may work together with other genes in an accumulated manner and result in an increased risk of esophageal tumor. DHPLC is a robust and sensitive technique for screening gene mutations.

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Key words: Mlh3; DNA mismatch repair; Familial esophageal cancer; Mutation screening; Denaturing high performance liquid chromatography

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Abstract

AIM: To shed light on the possible role of mismatch repair gene Mlh3 in familial esophageal cancer (FEC).

METHODS: A total of 66 members from 10 families suggestive of a genetic predisposition to hereditary esophageal cancer were screened for germline mutations in Mlh3 with denaturing high performance liquid chromatography (DHPLC), a newly developed method of comparative sequencing based on heteroduplex detection. For all samples exhibiting abnormal DHPLC profiles, sequence changes were evaluated by cycle sequencing. For any mutation in family members, we conducted a segregation study to compare its prevalence in sporadic esophageal cancer patients and normal controls.

RESULTS: Exons of Mlh3 in all samples were successfully examined. Overall, 4 missense mutations and 3 polymorphisms were identified in 4 families. Mlh3 missense mutations in families 9 and 10 might be pathogenic, but had a reduced penetrance. While in families 1 and 7, there was no sufficient evidence supporting the monogenic explanations of esophageal cancers in families. The mutations were found in 33% of high-risk families and 50% of low-risk families.

CONCLUSION: Mlh3 is a high risk gene with a reduced penetrance in some families. However, it acts as a low risk gene for esophageal cancer in most families. Muta-

INTRODUCTION

Esophageal carcinoma (EC) is one of the most common malignant tumors in China^[1,2]. Although various therapeutic strategies have been improved in recent years, the 5-year survival remains poor, therefore, early diagnosis and treatment of EC are still urgently needed^[3,4].

Current studies showed that multiple factors and steps contribute to its tumorigenesis, however the definite mechanisms remain to be determined yet^[5,6]. While in cancer families, genetic predisposition may play a more important role in carcinogenesis than other factors^[7]. Genetic susceptibility can be identified by detecting germline mutation of certain genes from peripheral blood.

Denaturing high performance liquid chromatography (DHPLC) is a newly developed technique suitable for the detection of heteroduplex mutations. DHPLC has the advantages of being highly automated, sensitive, and could be used for screening of gene mutations on a large scale^[8].

Great attention has been paid to mismatch repair (MMR) genes due to their association with hereditary non-polyposis colorectal cancer (HNPCC)^[9]. Mlh3 gene was first identified in 2000, which is a putative MMR gene normally expressed in multiple epithelia. It is located on 14q24.3 with a coding length of 4.3 kb, and is composed of 12 exons, of which exon 1 is 3.3 kb, accounting for 75%^[10]. Some investigations showed that it might play a certain role in the tumorigenesis of colorectal cancer^[11-14]. However, some studies indicate that there is no strong

association between currently known Mlh3 variants and colorectal cancer predisposition risk^[15,16]. A recent study on familial gastric cancer showed that Mlh3 can act as a low risk gene^[17].

Therefore, in an attempt to further evaluate its possible role in the cancer family, we performed mutation screening of Mlh3 in familial esophageal cancer (FEC) with DHPLC.

MATERIALS AND METHODS

Subjects

A total of 10 families with a hereditary background of esophageal carcinoma were collected (Table 1). The criteria were set as: at least 2 generations with esophageal cancers, at least two affected members, first-degree relatives, and carcinogenesis at earlier ages (< 50 years old). In addition to 66 members from these 10 esophageal families, 96 cases of sporadic esophageal cancer and 96 normal controls were recruited from the Department of Thoracic Surgery, First Hospital, China Medical University. Blood samples were collected from the members of each family after giving their informed consent.

DNA Extraction

DNA was extracted using the standard phenol/chloroform extraction protocols.

PCR

Seventeen primers were used for a total of twelve exons in Mlh3. Exon 1 was divided into seven overlapping fragments, and exons 8 and 9 were amplified together because of their small size and the small intron between them. The length of PCR fragments, primer sequences and corresponding annealing temperature for each fragment are shown in Table 2. All products were examined for specificity and quantity by 2% agarose gel electrophoresis.

DHPLC

PCR products were denatured at 95°C, and gradually cooled down to room temperature to enable efficient formation of heteroduplexes. DHPLC was carried out in a transgenomic wave DNA fragment analysis system, an automated DHPLC instrumentation equipped with a DNASep column. Abnormal elution profiles were identified by visual inspection of the chromatogram on the basis of the appearance of one or more additional earlier eluting peaks. Temperatures used in DHPLC analysis have been described elsewhere^[14].

Direct sequencing

Genomic DNA was re-amplified with DHPLC primers. After purification, DNA sequence changes were evaluated by direct sequencing in both directions. After treatment with ethanol, cycle sequencing products were analyzed on an ABI PRISM® 377 DNA sequencer.

Segregation study

For any specific variant of Mlh3 gene, we also detected its percentage in 96 cases of sporadic esophageal cancer and 96 normal controls in addition to the segregation study in

Table 1 Esophageal cancer family

Type	Family
TCR ¹	1, 5, 8, 10
MCR ²	2, 3, 4, 6, 7, 9

TCR¹: Two first-degree relatives with esophageal cancer; MCR²: Three or more relatives with esophageal cancer.

Table 2 Sequence, length and annealing temperature for primers

Primer sequence	Length (bp)	Annealing temperature (°C)
MLH3 F1-1: TCC AGT CAG AGA AGG AAA CCA	524	Touch down 58-51
MLH3 R1-1: ACA GGA AGC TGG TAA AAT AG		
MLH3 F1-2: CTG ATG TGA CTA GAG CAA GCG	552	Touch down 58-51
MLH3 R1-2: CAT CAT ACT CAC AGA ATT GGC AC		
MLH3 F1-3: ATT CAA GTC TTC GGC ACC	512	Touch down 58-51
MLH3 R1-3: TTT GTT TTG TAA AGA TGG CTC TG		
MLH3 F1-4: GGG ATT CAG AAG CTA CCA	602	Touch down 58-51
MLH3 R1-4: TGA ATG TTC TGT TTC AGT TGA TTT		
MLH3 F1-5: GGG CGA GIT AAA TTA TGT TCC A	562	Touch down 58-51
MLH3 R1-5: CTT GAA GAC TGA GAT TGG TAG TGA		
MLH3 F1-6: TGG GAA GGT TGA AAA TCC TC	557	Touch down 58-51
MLH3 R1-6: AGG AAT TAT CCT GTG TGG CAG		
MLH3 F1-7: CAG AGA ATG GTG TCA TCC CAA	551	Touch down 58-51
MLH3 R1-7: CCT TGT CCA GCA TTC CCA T		
MLH3 F2: TGT CTT GAC TCA GIT TGT GCA G	247	62
MLH3 R2: ACG ATG TGT ACT GTG TGC CC		
MLH3 F3: TGG TTC TGG ATG CCA ACT TT	229	59
MLH3 R3: ATT TCA GTC TGG GCA ACA GG		
MLH3 F4: CAA TTA TAT TTT GCT GAG TC	158	52
MLH3 R4: ATG AGA TTT TGA AGT TAA TC		
MLH3 F5: CCC AGT CTC AAA GAA AGG AGT	239	57
MLH3 R5: AGC TGG TTA GTC ATT CAG GC		
MLH3 F6: CAT GAT GGT TGT CGT CIT GC	185	60
MLH3 R6: GGT GTA CTG ATT CTG CTG GGA		
MLH3 F7: TTC CCT TCC TAC TCT TAA CCC A	265	59
MLH3 R7: TGT AAC CTC TCT TGG TCT CAT CTG		
MLH3 F8: TTT GGA ACC AGT AGT GAA GTG C	267	57
MLH3 R8: CAG CAA TTT CCT TAA CAT CTG C		
MLH3 F9/10: CGT AGA TTA AAG CCG ATT TTC	329	59
MLH3 R9/10: TGT ACC CTC TGC CTC TTT CG		
MLH3 F11: GTC AGC ATT GGT TTC CCA CT	251	59
MLH3 R11: AAA CIT TGC TCC CTC CTG CT		
MLH3 F12: GCC CAG CCT GTA TGC TAC CT	226	59
MLH3 R12: CAG TGA CAC TCC CTT TGT TCC		

families. If there was no such mutation in sporadic cancer or in normal controls, then this mutation might be pathogenic in this family. On the other hand, it might be a polymorphism not associated with the disease.

Table 3 Possible mutations of Mlh3 in familial esophageal cancer

Exon	Nucleotide change ¹	Amino change	Family	Family type	Cosegregation	Frequency in FEC <i>n</i> (%)	Frequency in sporadic EC <i>n</i> (%)	Frequency in normal control <i>n</i> (%)
1	A2173C	Asn725His	9	MCR	Yes	4/66 (6.1)	0/94 (0)	0/96 (0)
1	C2825T	Thr942Ile	10	TCR	Yes	3/66 (4.5)	1/95 (1.0)	0/96 (0)
7	T3826C	Trp1276Arg	1	TCR	No	3/66 (4.5)	0/96 (0)	0/96 (0)
12	G4335A	Gln1445Glu	7	MCR	No	6/66 (9.1)	0/95 (0)	0/96 (0)

¹ Numbering is according to the cDNA starting at the A in the start codon, Genbank AF195657.

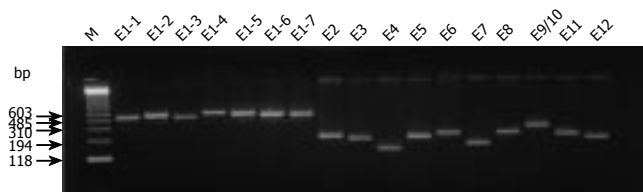


Figure 1 PCR results of hMLH3 fragments on 2% agarose gel electrophoresis. M: Molecular marker.

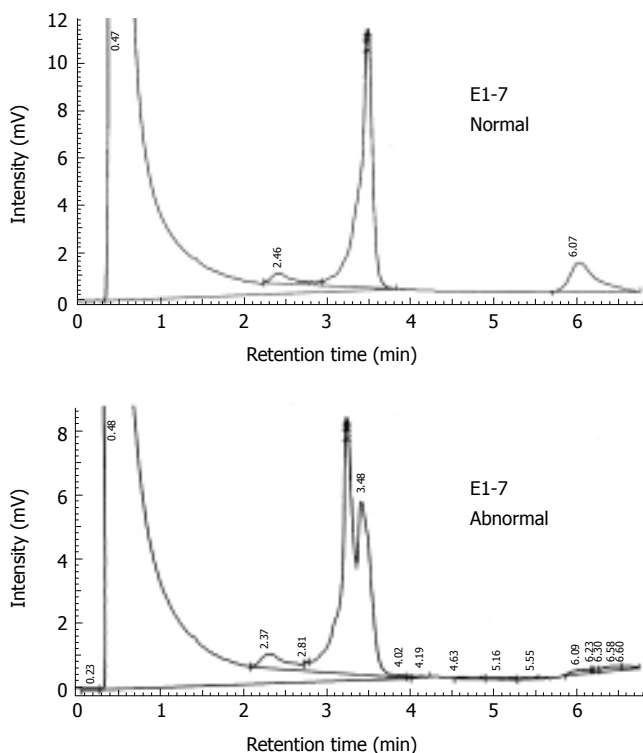


Figure 2 Normal and abnormal DHPLC chromatogram. The normal control appears as a clear elution peak, while one or more additional earlier eluting peaks result from heteroduplex for mutated DNA.

RESULTS

All the exons were successfully amplified (Figure 1), analyzed with DHPLC (Figure 2) and sequenced (Figure 3). Four missense mutations and 3 polymorphisms were identified in 4 families (Tables 3 and 4). One variant C2531T (Pro8441Leu) had almost the same prevalence in familial cancer, sporadic cancer and controls. One silent variant C2838A (Ser947Ser) and an intronic variant (between

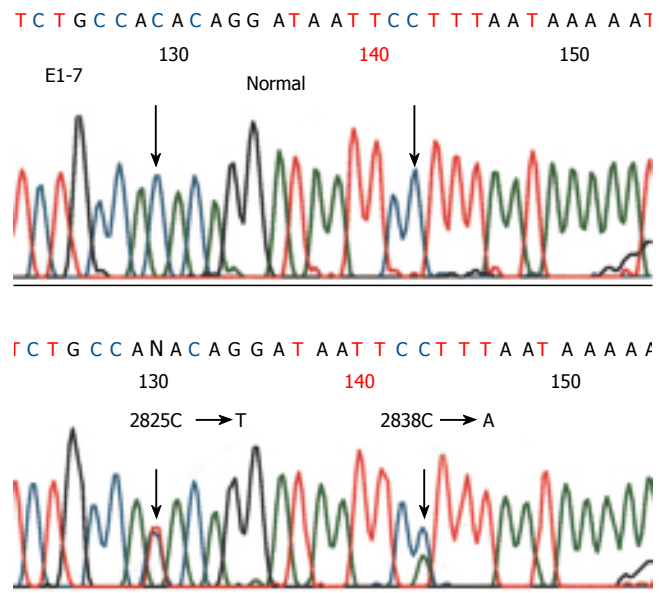


Figure 3 Sequencing results revealing two heterozygous variants in fragment 7 of exon 1, C2825T and C2838A. On the basis of amino acid variation, C2825T (Thr942Ile) is considered a missense mutation, while C2838A (Ser947Ser) a polymorphism.

Table 4 Polymorphism of Mlh3

Exon	Nucleotide change ¹	Amino change	Family
1	C2531T	Pro8441Leu	Many
1	C2838A	Ser947Ser	Many
9/10	IVS9 + 66A→G	Unknown	Many

¹Numbering is according to the cDNA starting at the A in the start codon Genbank AF195657.

exons 9 and 10) A→G, were considered polymorphisms (Table 4). All variants were not in the conserved homologous regions of the MutL protein at the NH₂ and COOH terminals, and none of the variants was evolutionarily conserved in yeast or *E. coli*^[18,19].

The families with identified mutations were of two types, high risk families consisting of more than three affected first degree relatives (MCR), and low risk families consisting of two affected first degree relatives (TCR) (Table 1). There were 6 high risk families and 4 low risk families. Two missense mutations (T3826C and C2825T) were found in two low risk families (families 1 and 10), the

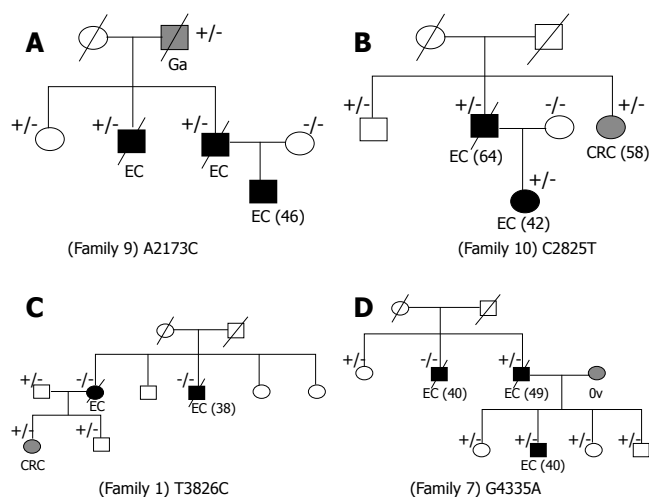


Figure 4 Pedigree of families with hMLH3 variants. Symbols and abbreviations used are denoted as black symbols for esophageal cancer, gray symbols for other cancers; EC: Esophageal cancer; CRC: Colorectal cancer; Ga: Gastric cancer; Ov: Ovarian cancer; Numbers next to the diagnosis denote age at onset; genotypes are on the top right of family member symbols; +: Variant carrier; -: Nonvariant carrier.

other two variants (G4335A and A2173C) were observed in high risk families (families 7 and 9). Mutations were found in 33% (2/6) of the high risk families and 50% (2/4) of the low risk families.

To elucidate the pathogenic nature of these 4 variants, we also detected their prevalence in sporadic cases and normal controls (Table 3). The data demonstrated that the frequency in families was far higher than that in sporadic cases and normal controls, suggesting that they might contribute to the disease in these families.

Missense mutation A2173C was found in 3 patients with esophageal cancer, one gastric cancer patient and one 76-year old unaffected relative in family 9 (Figure 4A), suggesting that the mutation could be inherited from the grandfather. Furthermore, no such variant was detected in controls and sporadic cases, suggesting that the mutation could be associated with the occurrence of esophageal cancer, but might have a reduced penetrance.

Missense mutation C2825T was found in family 10 (Figure 4B). The mutation was shared by the father and daughter with esophageal cancer, unaffected brother (66 years old) and sister with colon cancer, which was consistent with the mutation associated with the disease but having a reduced penetrance. Moreover, the mutation was found in 4.5% of family members and 1.0% of sporadic cases, but not in controls.

Variant T3826C (Trp1276Arg) in exon 7 was found in family 1 (Figure 4C). However, the mutation did not segregate well with the disease. Two affected members with esophageal cancer had no such mutation. While one female with colon cancer and two males with no colon cancer had this mutation. T3826C was not detected in controls and sporadic cases.

Variant G4335A was found in a male patient with esophageal cancer and his affected son in family 7, but not in his brother with esophageal cancer, whereas his unaffected sisters and three healthy children shared this mutation (Figure 4D). G4335A was not detected in sporadic

cases and normal controls.

Among the esophageal cancer families, Mlh3 mutation A2173C in family 9 and C2825T in family 10 could be causative, but had a reduced penetrance. These mutations were segregated with the disease, and neither of them was found in sporadic cases and normal controls. In variant T3826C in family 1 and G4335A in family 7, there was a lack of evidence of the monogenic high risk predisposition to esophageal cancer associated with these identified variants, because there was no clear association between mutation and the disease. However, these variants were more prevalent in families but not in sporadic cases and the controls, indicating a possible pathogenic nature in these families. Surprisingly, mutations were found in 50% (2/4) of low risk families and in 33% of high risk families (2/6).

DISCUSSION

The mismatch repair system is composed of a highly diverse group of proteins that interact with numerous DNA structures during DNA repair and replication^[20].

The MutL homologue, Mlh3 gene was first identified as a new member of the DNA MMR gene in yeast, and has been mapped to the region of the mouse complex trait locus, colon cancer susceptibility I^[10]. It is highly conserved in evolution. The protein encoded by its carboxyl-terminal may interact with the currently known mismatch repair gene MLH1, and is very similar to Mlh3p in yeast, indicating that it may play a role in the DNA mismatch repair system^[10]. It is essential not only for DNA repair and microsatellite stability, but also for meiosis. In *S. cerevisiae*, Mlh1-Mlh3 complex acts competitively in a distinct way to promote crossing over during meiosis along with the Msh4-Msh5 and Mus81-Mms4 complexes^[21]. The significant heterogeneity in localization of the MutL homologues, Mlh1 and Mlh3, directly results in defective crossing over during meiotic recombination in prophase I, and consequently 30% of human oocytes are predisposed to aneuploidy^[22].

Furthermore, Wu *et al*^[23] used knockout *mlh3* (-/-) mice to address the role of Mlh3 in class switch DNA recombination (CSR) and somatic hypermutation (SHM), and found that Mlh3 deficiency alters both CSR and SHM, suggesting that the MMR Mlh3 protein plays a role in both CSR and SHM.

A recent study reported that Mlh3 contributes to tumor suppression in mice. Mlh3 deficiency causes microsatellite instability, impaired DNA-damage response, and increased gastrointestinal tumor susceptibility^[24]. There is evidence that mismatch repair gene mutations is significantly associated with an increased risk of developing colorectal cancer^[25] and that the mismatch repair gene Mlh3 plays an important role in DNA repair.

There is evidence that mismatch repair gene Mlh3 plays an increasingly important role in DNA repair after replication, microsatellite stability, meiosis and tumor suppression.

In this study, among the 66 members in 10 esophageal cancer families, 4 missense mutations and 3 possible polymorphisms were identified. Even though all the

identified mutations were not in the conserved region and none of them changed a codon conserved in yeast and *E. coli*, their possible role in tumorigenesis cannot be ruled out. In fact, many reported missense mutations are not within the conserved region of hMLH1, another important mismatch repair gene^[26].

Current investigations have been mainly focused on its susceptibility to colorectal cancer (CRC)^[11-16]. By using DHPLC to screen Mlh3 mutations in 70 families with likely genetic predisposition to colon cancer, Liu *et al*^[14] found that the frequency of its germ-line variants, including one frameshift mutation, 10 missense mutations and 5 polymorphisms was high (23%). By analyzing 30 CRC cases for germline mutations by sequencing, Hienonen *et al*^[15] have found 5 missense variants, 4 of which were also found in cancer-free controls. The only remaining variant does not appear to be an attractive candidate for a disease-associated mutation because the amino acid change is located outside the conserved residues. Furthermore, they have not found the reported frameshift mutation in the 30 CRC cases or in 700 cancer-free controls. While it is a difficult task to exclude the role of Mlh3 in HNPCC, their study could not confirm the role of Mlh3 in CRC predisposition. Similarly, De Jong *et al*^[16] have found two variants (S845G and P844L) in Dutch patients with suspected HNPCC, but further investigation has failed to demonstrate an association between the two variants and colorectal cancer risk. Zhao *et al*^[17] performed an investigation on Mlh3 in 16 suggestive hereditary gastric cancer families with DHPLC and identified 5 missense mutations. However, no strong association has been verified between these 5 variants and gastric cancer risk, indicating that Mlh3 probably acts as a low risk gene in familial gastric cancer.

To establish whether the mutations segregate with disease in a family, we tried to collect DNA samples from members of each family. Two variants (A2173C and C2825T) showed a segregation pattern consistent with a monogenetic risk factor in families 9 and 10. Additionally, neither of them was detected among sporadic esophageal cancer cases and normal controls. However, because the sizes of these families were small, and the affected individuals were all first-degree relatives, Mlh3 could not be defined as a high penetrant predisposing FEC gene. The other variants (T3826C and G4335A) had no clear segregation pattern consistent with the disease, even the frequencies in families were higher than those in sporadic cases and controls.

Although the mutations of Mlh3 identified in our study did not provide sufficient evidence supporting a monogenetic explanation for the familial aggregation of EC, we would still like to propose that some or all of these mutations might work as low risk genes, perhaps in an accumulated manner, resulting in increased risk for EC. We believe that the majority of EC are caused by some low risk genes, which act on their own or interact in an additive manner. Therefore, the monogenetic susceptibility is still suggested for the disease in high risk families (family 7 and 9), while multiple genetic inheritance of susceptibility could be the reason in low risk families (family 1 and 10). Chen *et al*^[24] found mismatch repair Mlh3 and Pms2

double-deficient mice have tumor susceptibility, shorter life span, microsatellite instability, and DNA-damage response phenotypes. Al-Tassan^[27] found that in MYH, another DNA repair gene, several missense mutations act in a recessive manner and cause colorectal cancers in three sibs, with compound heterozygous missense mutations in this gene. Some mutations were also found as heterozygous missense mutations in healthy members of the same family and normal controls. Although, the predisposition seems to be inherited in a recessive manner, it is possible that heterozygous mutations in this gene also act in an additive manner as a low risk gene. And in our previous study on colorectal cancer families, we found a hMLH3 mutation segregated with disease together with a missense mutation in hMSH2^[14]. The missense mutations of both Mlh3 and Msh2 contributes to the failure of mismatch repair-mediated tumor suppression, resulting in the family aggregation of colorectal cancer.

Additionally, DHPLC analysis is very sensitive, robust and reproducible. Nevertheless, the relatively high expensive apparatus and related reagents have limited its wide use.

Based on previous reports and our results, the Mlh3 gene might occasionally appear as a high risk gene predisposing to EC, while in most cases it works as a low risk mismatch repair gene, contributing to the increased risk of developing EC. Despite our findings, much is yet to be learned about the molecular basis of correlations between genetic changes and clinical features of the disease. DHPLC is a sensitive and robust technique for screening gene mutations.

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Gene therapy that inhibits NF- κ B results in apoptosis of human hepatocarcinoma by recombinant adenovirus

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Abstract

AIM: To investigate whether the recombinant adenovirus induces the TNF- α -mediated apoptosis *in vivo*.

METHODS: Human hepatocarcinoma cell line (HepG₂) cells were transfected into BALB/c nude mice, and the tumor growth curve was drawn. We analyzed apoptosis in HepG₂ cells by TUNEL, HE staining and electron microscopy.

RESULTS: AdI κ B α M was expressed stably and efficiently in HepG₂ and could not be degraded by induction of TNF- α . Tumor growth in mice could be reduced remarkably if treated by AdI κ B α M plus TNF- α . There was apoptosis of > 70% of cells treated with AdI κ B α M plus TNF- α and about 50% of cells treated with AdI κ B α M. In contrast, there was few cell apoptosis in HepG₂ cells treated with phosphate buffered saline and AdI κ B α . HepG₂ cells in mice also exhibited a high level of apoptosis after *in vivo* injection with AdI κ B α M. The tumor growth curve indicated the tumor transfected with AdI κ B α M could be restrained.

CONCLUSION: AdI κ B α M gene therapy greatly enhances apoptosis due to inhibition of an NF- κ B-mediated antiapoptosis signaling pathway.

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Key words: NF- κ B; I κ B α ; Adenovirus

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INTRODUCTION

Resistance of tumor cells toward induction of apoptosis is one of the main reasons for failure of anticancer treatment^[1]. NF- κ B is a ubiquitous transcription factor that is activated by a variety of cytokines and mitogens^[2] and is thought to be a key regulator of genes involved in inflammation, response to infection, and stress. Classic NF- κ B is a heterodimer of p50 (NF- κ B-1) and p65 (Rel-A), but proteins that constitute the NF- κ B family form a variety of homodimers and heterodimers^[3]. NF- κ B is retained in an inactive form in the cytoplasm through association with one of the I κ B inhibitory proteins, including I κ B α , I κ B β and I κ B ϵ ^[4]. After cellular stimulation, the phosphorylation, ubiquitination, and subsequent proteolysis of I κ B α in proteasomes enables NF- κ B to translocate into the nucleus^[5-8], where it regulates the transcription of NF- κ B-response genes by interacting with κ B binding sites^[9,10]. Recently, abundant evidence has implicated cellular NF- κ B transcription factors in the control of apoptosis in many systems. It has been suggested to be associated with increased survival in many tumor cells. A number of studies implicated NF- κ B in apoptosis resistant tumor cells^[11-16]. A superrepressor form of I κ B α contains a serine-to-alanine mutation at amino acids 32 and 36, which inhibits signal-induced phosphorylation and subsequent proteasome-mediated degradation of I κ B α . This I κ B superrepressor has been used to demonstrate that inhibition of NF- κ B induces apoptosis through a variety of cancer therapeutic agents and TNF- α ^[17,18]. Based on this, we have successfully cloned the I κ B α gene and constructed the superrepressor I κ B α M in Chinese. We have generated recombinant adenovirus AdI κ B α M, which will provide a solid basis for the study of I κ B α -mediated antitumor gene therapy. In the present study, we investigated whether the recombinant adenovirus induces the TNF- α -mediated apoptosis in the human hepatocarcinoma cell line (HepG₂) and *in vivo*.

MATERIALS AND METHODS

Cell culture

HepG₂ cells, Hela cells and 293 cells were maintained in RPMI 1640 medium with 10% fetal bovine serum, penicil-

lin (100 mg/L), and streptomycin (100 mg/L).

Construction of recombinant adenovirus AdI κ B α M

The full-length cDNA of I κ B α superrepressor(I κ B α M), whose serines 32, 36 were mutated into the alanine, was kindly provided by Dr Bing-Rong Liu (from our laboratory). The I κ B α M was inserted into the adenoviral shuttle plasmid Track-CMV(a gift from Mr TC He, Molecular Oncology Laboratory, the University of Chicago Medical Center). It contains green fluorescent protein(GFP). We thus constructed the recombinant adenoviral plasmid pAdI κ B α M. However, pAdI κ B α did not have replacement of serines 32, 36 with alanines. Recombinant adenoviral plasmids were digested with *Pac* I. Then, the digested recombinant adenoviral plasmid was transfected into 293 cells with FuGENETM 6 transfection reagent(Roche). Viral transfection products (AdI κ B α M, AdI κ B α) were monitored by GFP expression. The recombinant adenovirus was selected and purified using standard procedures^[19]. To obtain a large quantity of recombinant adenovirus (AdI κ B α M, AdI κ B α), the 293 cells were infected and grown for 48 h at 37°C. The infected cells were harvested and centrifuged using a tabletop centrifuge at 1000 r/min for 5 min. The infected cells were resuspended in PBS. The cells were lysed by 4 freeze-thaw cycles to release the virus. The virus was purified through 1 CsCl gradient. The purified recombinant adenovirus was then titrated by the plaque assay^[19], aliquoted, and stored at -70°C until use.

HepG₂ cell analysis in BALB/c nude mice

All mice whose age was 4 wk, weighing between 15-18 g were provided by SLACCAS, China. Number of females was equal to males. Approximately 2×10^5 HepG₂ cells in 200 μ L of PBS media were injected subcutaneously into the back of 40 BALB/c nude mice. All mice were maintained and handled under specific pathogen-free conditions at the Animal Center in Chongqing University of Medical Sciences. The tumors of 8 mice in each group were directly injected with 2×10^9 plaque-forming units(PFU) of AdI κ B α M. Eight mice were injected with 2×10^9 pfu of AdI κ B α . The third group of mice was injected with 2×10^8 pfu of AdI κ B α M and the fourth group with 2×10^7 pfu of AdI κ B α M. Groups of control mice were injected with phosphate buffered saline (PBS). All mice were injected 5 times in total, every other day, 100 μ L each time. After the injection was finished, on the fourth day all mice were killed. The tumor growth curve was drawn. The volume of tumor was calculated according to the formula: Tumor volume = Length \times Width² \times 0.4.

Hematoxylin and eosin staining

HE staining analysis was carried out for evaluation of cell necrosis and apoptosis. The percentage of cells undergoing apoptosis was determined as the number of HE-positive cells in at least 10 randomly selected vision fields of sections obtained from tumors in each group of mice.

TUNEL analysis of HepG₂ cell apoptosis in BALB/c nude mice

HepG₂ cell apoptosis in BALB/c mice was determined

using *in situ* apoptosis staining with the TUNEL staining kit according to the manufacturer's instructions (Roche). Tissues from the mouse tumor were fixed in 4% buffered paraformaldehyde for 4 h and decalcified in 59 mmol/L EDTA, pH 7.8, for 3 wk. The tissue was then dehydrated with different concentrations of ethanol and xylene, and embedded in paraffin. Tissue specimens were cut into 8- μ m sections and mounted onto glass slides. Slides were incubated with fresh proteinase K (20 mg/L)-streptavidin-labeled horseradish peroxidase (HRP) at room temperature for 10 min. The slides were covered with a cover glass and incubated at 37°C for 1 h in a humidified chamber. Nonspecific staining was blocked by incubating the slides with blocking buffer at room temperature for 30 min. The slides were incubated with a klenow labeling buffer in the presence of biotin-labeled dNTP for 1.5 h at 37°C. After washing 6 times with PBS, the slides were incubated with streptavidin-conjugated antibody at a 1:50 dilution in Tris buffer, pH 7.4 with PBS and developed by incubation with diaminobenzidine solution for 5 min. Cells undergoing apoptosis were identified by dark brown staining of the nuclei. For quantitative analysis of the percentage of apoptotic cells, a total of 10 random vision fields were evaluated.

Electron microscopy

Electron microscope analysis was carried out for evaluation of cell necrosis and apoptosis.

Statistical analysis

Student's *t*-test was used for testing the statistical significance of the differences between the groups. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Construction and analysis of recombinant adenovirus AdI κ B α M

The recombinant adenovirus plasmids were generated by cloning the I κ B α M/I κ B α construct into adenoviral shuttle plasmid Track-CMV. The recombinant adenovirus plasmids were digested with *Pac* I. The digested products were identified by 0.8% agarose gel electrophoresis (Figure 1A). pAdI κ B α M was digested into 2 fragments: one was about 3 kb, the other probably 30 kb; pAdI κ B α was digested into fragments of about 4.5 kb and 30 kb. The digested recombinant adenovirus plasmid was transfected into 293 cells. Two days after transfection, the fluorescence was observed (Figure 1B, C). The results showed that we have successfully cloned the I κ B α M/I κ B α gene into the plasmid Track-CMV, and the recombinant adenovirus AdI κ B α M/AdI κ B α was established. The virus was grown to a titer of 2×10^{12} pfu/L by purification over a CsCl gradient. AdI κ B α M could be expressed in HepG₂ cells after infection for 48 h (Figure 1D).

Induction of apoptosis of HepG₂ in BALB/c nude mice by AdI κ B α M *in vivo*

HepG₂ cells (2×10^5) were injected into the back of 40 BALB/c nude mice subcutaneously. After 2 wk, a tumor

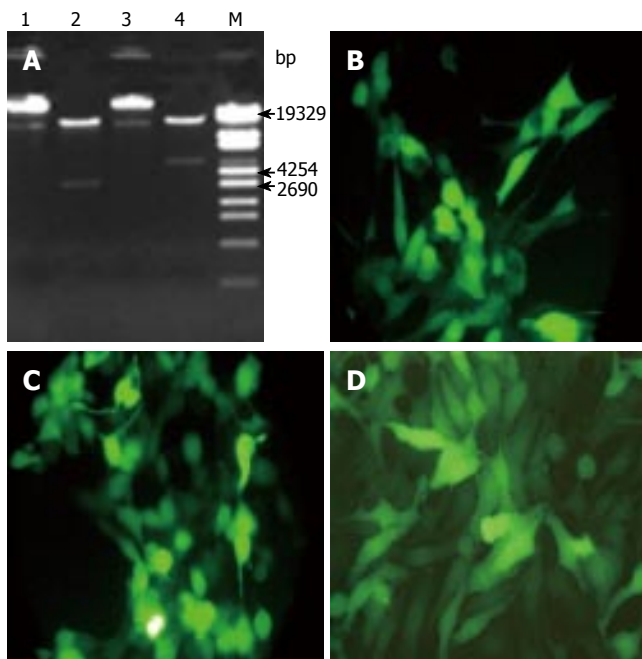


Figure 1 M: DNA Marker; Lanes 1, 3: Recombinant adenoviral plasmids pAd-IκBαM/pAd-IκBα un-cleaved with *Pac I*; Lanes 2, 4: Recombinant adenoviral plasmids pAd-IκBαM/pAd-IκBα cleaved with *Pac I*. **A**: Identification of recombinant adenoviral plasmids by restriction analysis; **B**, **C**: The pAd-IκBαM/pAd-IκBα was transfected into 293 cells and GFP expression was observed by fluorescence microscopy after transfection for 36 h; **B**: pAd-IκBαM; **C**: pAd-IκBα ($\times 200$). **D**: The recombinant adenovirus AdIκBαM infected into HepG2 and GFP expression was visualized by fluorescence microscopy after transfection for 48 h, ($\times 200$).

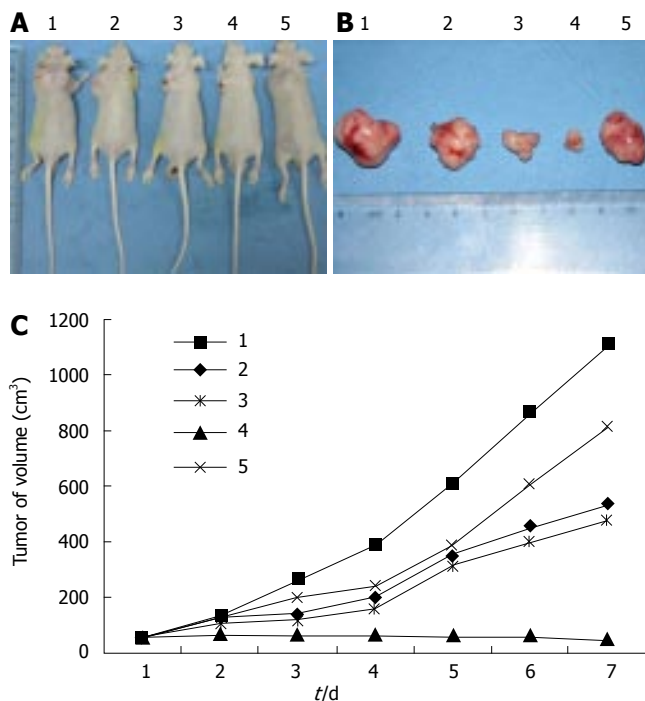


Figure 2 **A**: Mice were killed on the fourth day after different injections (1: Control PBS, 2: AdIκBαM 2×10^{10} , 3: AdIκBαM 2×10^{11} , 4: AdIκBαM 2×10^{12} , 5: AdIκBα 2×10^{12}); **B**: Tumors from mice were processed for histologic analysis. The sequence of mice is according to that in **A**; **C**: The tumor growth curve. The sequence of mice is according to that in **A**, **B**.

about 5 mm was observed in all mice. All mice were

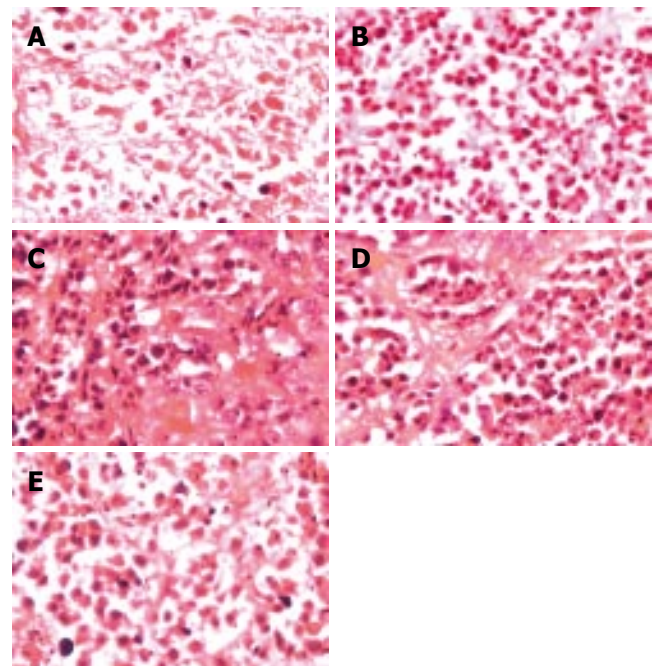


Figure 3 Morphological changes of HepG2 cells from mice treated with different methods were analyzed by HE staining. **A**: PBS control; **B**: AdIκBαM (2×10^{10}); **C**: AdIκBαM (2×10^{11}); **D**: AdIκBαM (2×10^{12}); **E**: AdIκBα (2×10^{12}).

killed on the fourth day after a different treatment in the different groups (Figure 2A). The tumors from all mice were processed for histologic analysis (Figure 2B). Tumor volumes were calculated (Figure 2C). No effect was observed in mice injected with control PBS. In contrast, BALB/c nude mice treated with AdIκBαM (2×10^{12} pfu/L) exhibited the most obvious inhibition of tumor growth, the tumor growth being stopped. Injection with AdIκBα (2×10^{12} pfu/L) had a slight effect on tumor growth at first, which then diminished.

HE staining and TUNEL analysis

There was a difference in the incidence of cell destruction by HE staining, which was exhibited by almost all mice (Figure 3). BALB/c mice injected with AdIκBα demonstrated a slight effect. In contrast, BALB/c mice treated with AdIκBαM (2×10^{12} pfu/L) exhibited extensive pyknotic nuclei and cell destruction. There were masses of cell necrosis in BALB/c mice injected with PBS, because ischemia in tumor resulted in cell necrosis. The number of apoptotic cells in the AdIκBαM (2×10^{12}) group was 16.8 ± 3.1 ($P < 0.01$); in the AdIκBαM (2×10^{11}) group 13.1 ± 2.3 ($P < 0.01$); in the AdIκBαM (2×10^{10}) group 10.1 ± 2.1 ($P < 0.01$); in the AdIκBα group 5.3 ± 1.8 ($P > 0.05$); in the PBS control group 3.8 ± 1.8 ($P > 0.05$). Moreover, to determine if AdIκBαM induced apoptosis *in vivo*, the tumor was sectioned and analyzed by *in situ* TUNEL staining (Figure 4). There was significant apoptosis of HepG2 cells infected with AdIκBαM (2×10^{12} pfu/L) but not of HepG2 treated with control PBS and some apoptosis in HepG2 cells injected with AdIκBα. Furthermore, there was a direct correlation between AdIκBαM dosage and cell apoptosis. The number of apoptotic cells in the AdIκBαM (2×10^{12}) group was 14.7

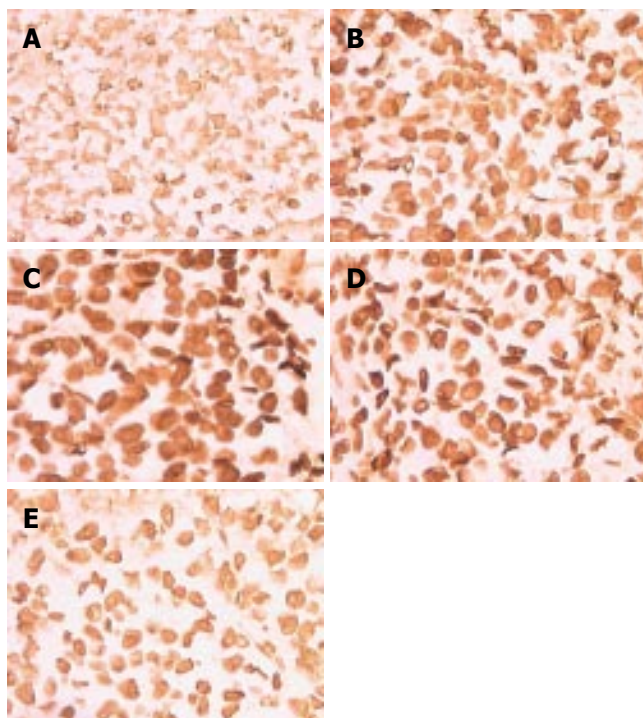


Figure 4 TUNEL was used to evaluate apoptosis of HepG₂ cells in BALB/c mice. **A:** PBS control; **B:** AdIkB α M (2×10^{10} pfu/L); **C:** AdIkB α M (2×10^{11} pfu/L); **D:** AdIkB α M (2×10^{12} pfu/L); **E:** AdIkB α (2×10^{12} pfu/L).

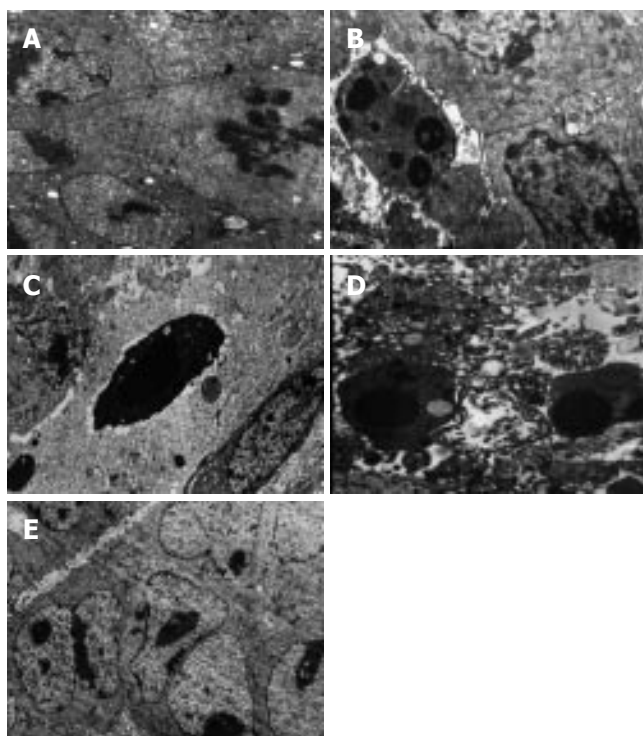


Figure 5 Electron microscopic analysis was used to evaluate apoptosis in HepG₂ cells in mice. **A:** PBS control ($\times 4000$); **B:** AdIkB α M 2×10^{10} pfu/L ($\times 6000$); **C:** AdIkB α M 2×10^{11} pfu/L ($\times 6000$); **D:** AdIkB α M 2×10^{12} pfu/L ($\times 6000$); **E:** AdIkB α 2×10^{12} pfu/L ($\times 4000$).

± 2.4 ($P < 0.01$); in the AdIkB α M (2×10^{11}) group 12.4 ± 2.2 ($P < 0.01$); in the AdIkB α M (2×10^{10}) group 8.3 ± 2.0 ($P < 0.01$); in the AdIkB α group 3.4 ± 1.6 ($P > 0.05$); in the PBS control group 4.2 ± 1.7 ($P > 0.05$).

Results of electron microscopy

Those cells with dark concentrated nuclei under EM were considered as apoptotic cells. In the AdIkB α M (2×10^{12}) group, there were 1-2 apoptotic cells in a random vision field ($\times 4000$ - 6000 , Figure 5A). Necrotic tissue or cells were observed around the apoptotic cells, but no proliferative phase was found. In the AdIkB α M (2×10^{11}) group, there were 0-2 apoptotic cells in a random vision field ($\times 4000$ - 6000 , Figure 5B). In the AdIkB α M (2×10^{10}) group, apoptotic cells could seldom be found (Figure 5C), whereas in the PBS control and AdIkB α group, there were few apoptotic cells, and many tumor cells were in the proliferative phase; however, necrotic tumor cells could also be found (Figures 5D, E). Necrotic tumor cells could be found in the AdIkB α M (2×10^{12}) group, probably because AdIkB α M could induce the tumor cells to apoptosis, and then to necrosis. Necrotic tumor cells could be found in the PBS control group probably because the tumor developed too fast and the blood supply was inadequate. Because the results of electron microscopy are the most reliable evidence of apoptosis, we could speculate that recombinant adenovirus could induce apoptosis of human hepatocarcinoma and inhibit the tumor cell proliferation.

DISCUSSION

TNF- α is an important cytokine in the promotion of growth and invasion of cells. It interacts with TNFR I and TNFR II. Signaling through both TNFR I and TNFR II can induce apoptosis^[20-22]. Interactions with TNFR I produce a proapoptotic signal by recruitment of TNFR I-associated death domain (TRADD) protein to the death-inducing signaling complex (DISC) of the TNFR I timer^[23,24]. TRADD recruits the Fas-associated death domain (FADD), which in turn, recruits caspase 8 and signals apoptosis^[25]. Simultaneously, an anti-apoptosis pathway involves recruitment of cellular LAP (cLAP), receptor interactive peptide (RIP), and TNFR-associated factor 2 (TNFR2), which leads to activation of NF- κ B-inducing kinase (NIK)^[26]. This results in phosphorylation of I κ B α and I κ B β and translocation of NF- κ B to the nucleus. This second signal predominates in hepatocarcinoma, and NF- κ B translocation to the nucleus plays a role in transcription of several genes, including TNF- α , interleukin-1 β , and IL-6, as well as collagenase, stromelysin, and adhesion molecules^[27,28]. At the same time, NF- κ B translocation inhibits apoptosis in many cell types, including tumor cell lines. HepG₂ cell line, like other cell lines, does not undergo apoptosis in response to TNF- α . Therefore, we propose that TNF- α acts as a growth factor in the HepG₂ cell line, as well as induces production of cytokines and invasive enzymes. Taken together, it implies that NF- κ B may play a role in preventing maximal apoptotic killing in treatment regimens such as TNF, radiation therapy, and certain chemotherapeutic agents. Resistance to anticancer therapies appears to be mediated by resistance to apoptosis. Therefore, modulation of NF- κ B activity could potentially lead to improved cell killing in the HepG₂ cell line and *in vivo*. Based on this understanding, we have successfully constructed the superrepressor of NF- κ B (AdIkB α M), in which 32, 36

serines were replaced with alanines and could not be phosphorylated by NIK.

The studies by Duffey *et al.*^[29] demonstrated that human head and neck squamous carcinoma cell transfected with I κ B α M was significantly restrained. The results of our study with AdI κ B α M showed that the HepG₂ cell line could be sufficiently infected with this recombinant adenovirus. Furthermore, I κ B α M could be expressed stably in HepG₂ cells. This was especially prominent with induction by TNF- α . Overexpression of mutated I κ B α by an AdI κ B α M construct resulted in inhibition of nuclear translocation of NF- κ B after TNF- α stimulation of HepG₂ cells. Under these conditions, the HepG₂ cell underwent extensive apoptosis in response to TNF- α *in vitro*. There was distinct apoptosis in HepG₂ cells treated alone with AdI κ B α M and little apoptosis treated with AdI κ B α and no apoptosis treated with PBS. This indicated that AdI κ B α M could facilitate or induce apoptosis in HepG₂ cells *in vitro*. There are histocyte cells and T cells in BALB/c nude mice, and the BALB/c mice may contain TNF- α *in vivo*. In light of the results of the experiment *in vitro*, we did not use TNF- α *in vivo*. BALB/c mice treated with AdI κ B α M (2×10^{12} pfu/L) exhibited the most extensive inhibition of tumor growth. In contrast, no effect was observed in mice injected with PBS and a slight effect in mice treated with AdI κ B α . Mice treated with AdI κ B α M (2×10^{12} pfu/L) underwent extensive apoptosis *in vivo*. However, there was little apoptosis in mice treated with AdI κ B α (2×10^{12} pfu/L). In this study, we focused particularly on the suppression of tumor growth and the induction of cell apoptosis by the recombinant adenovirus. Although various degrees of necrosis could be observed by HE staining, more work should be done on whether the recombinant adenovirus could lead to tumor death through inducing the damage of tumor blood vessels. Meanwhile, we should pay more attention to the toxicity of recombinant adenovirus, the first-pass effect of liver, the antigenicity and the targeting of recombinant adenovirus.

In conclusion, the AdI κ B α M is expressed in HepG₂ cell effectively and stably. It could inhibit the activity of NF κ B, and cause increased apoptosis as well as suppression of liver tumor.

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Triple antiviral therapy in HCV positive patients who failed prior combination therapy

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Abstract

AIM: To assess the efficacy of triple therapy (peginterferon or high dose standard interferon, plus ribavirin and amantadine) in nonresponders to prior combination therapy.

METHODS: A total of 196 patients were enrolled in a multicenter, open, randomized study. Patients were given 180 µg/wk of peginterferon-alpha-2a (40 kDa) plus ribavirin (800-1000 mg/d) and amantadine (200 mg/d) for 48 wk (group A) or interferon-alpha-2a (6 MU/d for 4 wk, 3 MU/d for 20 wk, and 3 MU tiw for 24 wk) plus ribavirin (800-1000 mg/d) and amantadine (200 mg/d) for 48 wk (group B).

RESULTS: Overall sustained virologic response (SVR) was 26.6% (32.1% and 19.5% in group A and B, $P = 0.057$). Baseline ALT >120 UI/L (OR 2.4; 95% CI:1.11 to 5.20; $P = 0.026$) and HCV RNA negativity after 12 wk (OR 8.7; 95% CI: 3.87 to 19.74; $P < 0.0001$) were independently associated with SVR. Therapy discontinuation occurred less frequently in patients treated with peginterferon than standard interferon ($P = 0.036$).

CONCLUSION: More than 25% of nonresponders to combination therapy can eradicate HCV infection when retreated with triple therapy, especially if they have a high baseline ALT and are treated with pegylated interferon.

INTRODUCTION

While advances in the treatment of HCV chronic hepatitis have markedly improved outcomes for treatment-naïve patients, a large number of patients still fail to eradicate HCV infection^[1-4], and improving the re-treatment success rates of these nonresponsive patients remains a key challenge in hepatitis care^[5-7]. It has been reported that 12%-20% of patients who did not respond to standard interferon (IFN) monotherapy can achieve a sustained response when retreated with high doses of interferon plus ribavirin (RBV)^[8-12]. However, the most effective treatment strategies for patients who fail to respond to combination therapy have yet to be established, and results for re-treatment with pegylated interferon and ribavirin in these patients are generally more variable and less positive^[13-17]. The results can also be difficult to interpret since most of the available data derive from studies that included both relapsers and nonresponders to different therapeutic schedules. However, considering the high number of patients who do not respond to combination therapy, the need for new antiviral re-treatment regimens becomes evident.

It has recently been suggested that the addition of amantadine (AMA) to interferon and ribavirin (triple therapy) may improve the therapeutic efficacy of combination therapy, possibly by potentiating the antiviral activity through interleukin production^[18]. Amantadine has an intrinsic antiviral activity against influenza A virus by blocking its cellular internalization and possibly interferes with the replication of other viruses including HCV^[19-21]. Results on the use of triple therapy in patients with HCV chronic hepatitis are conflicting. A recent meta-analysis^[22] reported that triple therapy was of no advantage compared to standard combination therapy in treatment-naïve

patients or in relapsed patients^[23,24], whereas it markedly improved the sustained virologic response in nonresponders to interferon monotherapy^[25-27]. The efficacy of triple therapy in nonresponders to combination therapy has primarily been investigated in only a small series of patients, making it difficult to draw defined conclusions from these data on the use of triple therapy in such patients^[28-32]. A recent study in a larger patient population of nonresponders ($n = 200$) found a higher sustained virological response in patients receiving triple therapy (24%) *vs* combined therapy (16%), although the difference was not statistically significant^[33].

The aims of this multicenter, randomized study carried out in a well-selected series of patients nonresponsive to combination therapy were (1) to define the rate of sustained virological response in patients previously nonresponsive to combination therapy, when retreated with triple therapy (consisting of recombinant interferon or peginterferon (PegIFN) plus ribavirin and amantadine); (2) to assess whether PegIFN is superior to a high daily dose of IFN when used in a triple therapy regimen; and (3) to compare the tolerability, side effects and adherence to therapy in patients treated with the two different schedules.

MATERIALS AND METHODS

From February 2001 to June 2002, 196 patients aged between 18 and 65 years were included in this multicenter cooperative study. All patients had to be nonresponders to interferon plus ribavirin given for at least 24 wk. Patients who had previously been treated with interferon alone or who had received combination therapy for a shorter period were excluded from the study. Patients were recruited by 21 Liver Centers operating in 21 General Hospitals sited in Lombardy (North of Italy) and belonging to the Gruppo Epatologico Lombardo (GEL). A noncompetitive recruitment was employed and each center had to enroll at least 5 patients. The failure to respond to previous combination therapy was defined as absence of normalization of transaminases and/or detectable serum HCV RNA level after 24 wk of therapy. A washout period of at least 6 mo from the end of therapy to inclusion in this study was required. For inclusion, patients had to fulfill the following criteria: (1) persistent transaminase elevation throughout the previous 6 mo; (2) a liver biopsy taken within the past 18 mo showing histological findings compatible with chronic hepatitis or cirrhosis; (3) serum HCV RNA positive by quantitative polymerase chain reaction (PCR) test (second-generation Amplicor HCV, Roche Diagnostic System, Basel, Switzerland). Patients were excluded from the study if one of the following criteria were present: (1) decompensated cirrhosis; (2) co-infection with human immunodeficiency virus; (3) positivity for HBsAg; (4) autoimmune hepatitis; (5) alcohol abuse (daily alcohol intake > 60 g in males and > 40 g in females); (6) haemoglobin concentration less than 120 g/L in women and 130 g/L in men; (7) white cell count less than 3×10^9 /L, platelet count below 100×10^9 /L; (8) moderate/severe depression or other psychiatric diseases; (9) seizure disorders; (10) cardiovascular, respiratory or renal clinically manifested diseases; (11)

hemoglobinopathies; (12) poorly controlled diabetes mellitus; (13) immunologically mediated disease; (14) ultrasonographic evidence of focal liver lesions.

Study design

This was a multicenter, open-label, randomized, parallel-group study. Randomization was centralized and patients were stratified by HCV genotype (genotype 1 *vs* other genotypes).

Patients were randomized to receive peginterferon- α -2a (Pegasys; Roche, Basel, Switzerland) at the dose of 180 mcg once a wk for 48 wk (Treatment A) or interferon alfa-2a (Roferon; Roche) at the dose of 6 MU/d for 4 wk followed by 3 MU/d for the next 20 wk and 3 MU tiw until the end of the study (wk 48) (Treatment B). Both groups received ribavirin (Copegus, Roche, Basel, Switzerland) at the dose of 800 mg (for patients weighing less than 75 kg) or 1000 mg (for patients weighing 75 kg or more) and amantadine (Mantadan; Boehringer Ingelheim, Florence, Italy) at a total daily dose of 200 mg. The treatment period lasted 48 wk, with a 24-wk follow-up period. Sustained virological response was defined as persistent negative HCV RNA at 6 mo after completion of therapy. Patients were considered nonresponders and therapy was stopped if HCV-RNA was still positive at 24 wk of therapy.

Basal laboratory assessment consisted of liver function tests, hematologic tests, renal function tests, autoantibodies (ANA, AMA, ASMA, antiLKM), alphafetoprotein, serum iron, transferrin and ferritin, HBsAg, anti-HBc. Quantitative HCV RNA was measured by standardized PCR assay with a lower limit of detection of less than 1×10^6 copies/L (second-generation Amplicor HCV Monitor, Roche), qualitative HCV RNA was measured by a standardized PCR assay with a lower limit of detection of 0.1×10^3 copies/L (second-generation Amplicor HCV, Roche Diagnostic System). Determination of infecting HCV genotype was performed in serum samples prior to therapy using Innolipa, Genetics Technique (Innogenetics, Belgium/Bayer Diagnostics, USA).

Patients were clinically evaluated at regular intervals: 15, 30, 45 and 60 d after the first dose of treatment and every month thereafter until the end of the study. Biochemical tests were performed at 15 and 30 d and at monthly intervals until the end of treatment and every 3 mo during follow-up. During treatment, HCV-RNA was assayed after 12, 24, 36, and 48 wk of therapy and after 12 and 24 wk from the end of the treatment. At 12 and 72 wk, the serum HCV RNA level was assayed by qualitative analysis.

Histology

Liver biopsies were formalin fixed, embedded in paraffin and stained by hematoxylin-eosin, reticulin and Masson's trichrome method. Histologic diagnosis was made by one independent pathologist and classified according to the internationally accepted criteria as chronic hepatitis (mild, moderate or severe) or cirrhosis. Inflammatory activity (grading) and fibrosis (staging) were semi-quantified using the Ishak scoring system^[34]. Local institutional committees of participating hospitals approved the study protocol and all amendments. All patients provided written informed

consent. All the study procedures were in accordance with the principles of the Helsinki Declaration.

Primary and secondary endpoints

Clearance of HCV RNA at the end of the 6-mo follow up period was the primary end-point. Safety, adherence to therapy and therapy interruption were considered as secondary end-points.

Safety assessment

Safety was assessed by physical examinations, laboratory tests and spontaneous reports of clinical adverse events. According to the study protocol, Peginterferon- α -2a dose modification to 135, 90 or 45 mg/wk and ribavirin dose reductions were allowed in patients with clinically significant adverse events or laboratory abnormalities, including hematological toxicity as manifested by the hemoglobin level of less than 100 g/L, white cell count of less than 2.5×10^9 /L, granulocyte count of less than 1×10^9 /L, and platelet count of less than 70×10^9 /L. Therapy was discontinued in patients with neutrophils below 0.7×10^9 /L, Hb below 85 g/L, and platelets below 50×10^9 /L. Adherence to treatment was considered when drug consumption (either pegylated/standard IFN or ribavirin or both) was higher than 70% of the scheduled dose for a time period longer than 70% of the scheduled time.

Statistical analysis

It was calculated that at least 100 patients per group were needed to detect a difference of 20% in the proportion of SVR between treatment A and treatment B with an alpha error of 0.05 and study power (beta error) of 0.8. Summary statistics (no. of cases, mean, median, standard deviation, minimum and maximum) were calculated for continuous variables, and the number and percentage of patients in each category were provided for categorical data. The treatment comparison of interest was the recombinant interferon- α -2a plus ribavirin plus amantadine against peginterferon- α -2a plus ribavirin plus amantadine. Differences between the treatments were assessed by the Cochran-Mantel-Haenszel test stratified according to HCV genotype (HCV genotype 1 *vs* other genotypes). The relationship between patients' baseline characteristics and SVR was examined by logistic regression analyses. Univariate logistic regressions were used to confirm the importance of previously identified prognostic factors. To assess the independence of these factors, a backward elimination procedure was then undertaken using the factors that were significant in the univariate analyses. A log-linear model was used to investigate the interactions between HCV-RNA eradication after 12 wk of therapy and the variables predictive of sustained virologic response. All *P* values reported are two-sided.

All patients who received at least one dose of study medication were included in all efficacy analyses, and if they had undergone at least one safety assessment after baseline, they were included in the safety analysis. Patients with missing data in the primary or secondary efficacy endpoints were considered as nonresponders.

Table 1 Baseline characteristics of patients (Information are not available for all patients) *n* (%)

Characteristic	All (<i>n</i> = 188)	PegIFN + RBV + AMA (<i>n</i> = 106)	IFN + RBV + AMA (<i>n</i> = 82)
Male/Female	146/42	79/27	67/15
Age			
≤ 40 yr	42 (22)	23 (22)	19 (23)
> 40 yr	146 (78)	83 (78)	63 (77)
BMI			
≤ 25 kg/m ²	107 (64)	60 (65)	47 (63)
> 25 kg/m ²	61 (36)	33 (35)	28 (37)
ALT			
≤ 120 UI/L	107 (62)	64 (65)	43 (58)
> 120 UI/L	66 (38)	35 (35)	31 (42)
HCV genotype			
1	147 (78)	82 (77)	65 (79)
2	21 (11)	12 (11)	9 (11)
3	8 (4)	7 (7)	1 (1)
4	12 (6)	5 (5)	7 (9)
HCV RNA			
$\leq 2 \times 10^6$ copies/mL	74 (73)	41 (76)	33 (69)
$> 2 \times 10^6$ copies/mL	28 (27)	13 (24)	15 (31)
Stage			
≤ 3	127 (69)	71 (70)	56 (68)
> 3	56 (31)	30 (30)	26 (32)
Grade			
≤ 10	167 (92)	94 (91)	73 (92)
> 10	15 (8)	9 (9)	6 (8)

RESULTS

Two hundred thirty two consecutive nonresponder patients to combination therapy were considered eligible for this study. Thirty six patients were not randomized because of refusal of therapy (23 cases) or inability to attend a regular follow up (13 cases). One hundred six patients were randomized to receive peginterferon- α -2a plus ribavirin and amantadine and 90 to receive interferon α -2a plus ribavirin and amantadine. Eight patients randomized to treatment B refused to take the study drugs and were not further evaluated (Figure 1). The characteristics of patients at baseline are shown in Table 1. None of the variables considered was statistically significantly different between the two groups.

A SVR of 26.6% was obtained in the overall series of patients. However, when the two treatments were compared, a better but not significant SVR was achieved in patients receiving PegIFN than in those receiving high doses (daily doses for 6 mo) of standard interferon (32.1% *vs* 19.5%, *P* = 0.057). The better response in patients treated with PegIFN was present at all on-treatment time intervals (Figure 2). The rate of sustained response in the 30 patients with cirrhosis was 30% with no difference between patients treated with PegIFN or standard IFN.

Univariate analysis performed on non-missing data, considering the overall series of patients, showed that genotype non 1, higher grade score and baseline ALT were significantly associated with sustained virologic response (Table 2). Patients younger than 40 years had a significantly better response when treated with PegIFN than standard IFN (48% *vs* 11%, *P* = 0.017). Also patients with fibrosis less than 3 and those with viremia higher than 2 million

Table 2 Sustained virologic response for all treatment groups combined according to the baseline characteristics of patients

Characteristics	Sustained virologic response		P ¹
Genotype 1	32/147	22%	0.0085
Non 1	18/41	44%	
Age ≤ 40 yr	13/42	31%	NS
> 40 yr	37/146	25%	
BMI ≤ 25 kg/m ²	32/107	30%	NS
> 25 kg/m ²	12/61	20%	
Stage ≤ 3	37/127	29%	NS
> 3	12/56	21%	
Grade ≤ 10	42/167	25%	0.03
> 10	8/15	53%	
ALT ≤ 120 UI/L	22/107	21%	0.03
> 120 UI/L	24/66	36%	
HCV-RNA ≤ 2 × 10 ⁶ copies/mL	23/74	31%	NS
> 2 × 10 ⁶ copies/mL	7/28	25%	
Previous Total IFN ≤ 500 MU	20/91	22%	NS
> 500 MU	25/82	30%	

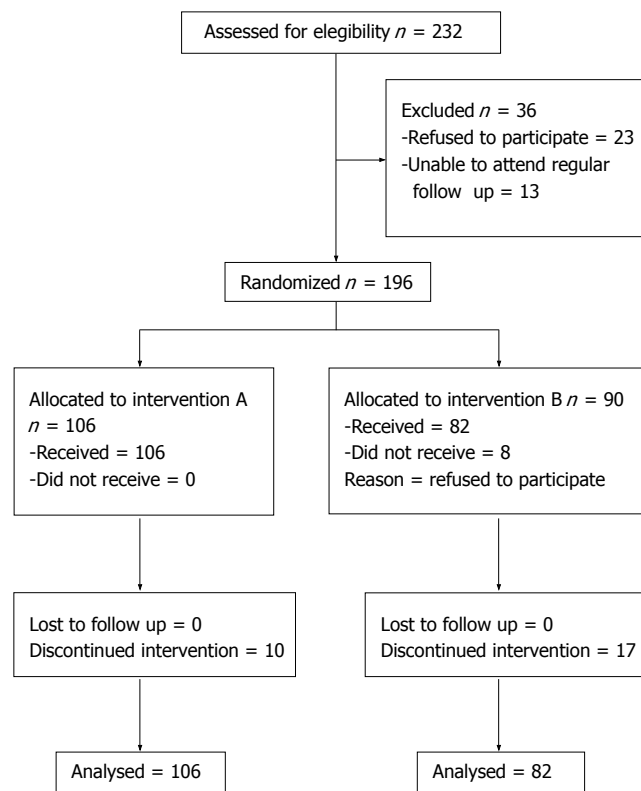
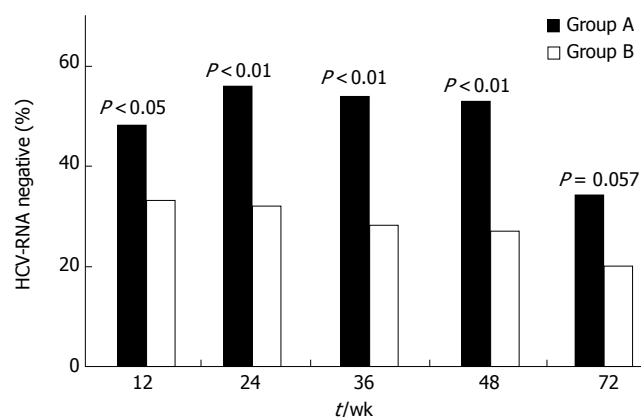
¹ Fisher's exact test; NS: Not significant.

copies had a significantly better response when treated with PegIFN than standard IFN (respectively 38% *vs* 18%, $P = 0.018$ and 46% *vs* 7%, $P = 0.029$) (Table 3).

The chance of achieving a sustained response was significantly higher in patients who were HCV RNA negative as compared to those who were HCV RNA positive at 12 wk of therapy (39/78, 50% *vs* 11/110 10%, $P < 0.0001$).

To examine the influence of potentially important prognostic factors on SVR, factors known to affect response (HCV genotype, age, BMI, stage and grade, ALT value, previous IFN total dose taken, biochemical and virologic response after 12 wk of treatment) were first examined individually by univariate logistic regression analysis for each factor for all treatment groups combined. ALT (< 120 UI/L *vs* > 120 UI/L), the biochemical and the virologic response at wk 12 were associated with SVR ($P < 0.10$). These predictive factors were entered in the final backward regression analysis. Two factors independently and significantly increased the odds of achieving a sustained virologic response: a baseline ALT value greater than 120 UI/L (odds-ratio 2.40; 95% CI: 1.11 to 5.20; $P = 0.026$), and the virologic response after 12 weeks of therapy (odds-ratio 8.75; 95% CI: 3.87 to 19.74; $P < 0.0001$). Logistic regression analysis was used to characterize further the relation between SVR, patients' characteristics at baseline, biochemical and virologic response after 12 wk of treatment, and the study treatments. In the final backward regression analysis were entered the treatment given, the ALT value at baseline, the biochemical and the virologic response after 12 wk of treatment. The model confirmed that the baseline ALT value greater than 120 UI/L and the virologic response after 12 wk of treatment were prognostic factors that significantly increased the odds of achieving a sustained virologic response. Odds-ratios and their 95% confidence intervals and the associated p-values were equal to those obtained in the logistic regression model reported above.

Twenty-seven patients discontinued the study treatments: 10 in group A and 17 in group B (9.4% *vs*

**Figure 1** Progress of patients through the trial showing details of patients' recruitment, enrollment, randomization and therapy withdrawal until completion of the study.**Figure 2** Comparison of virologic responses over time in patients treated with peginterferon plus ribavirin and amantadine (Group A), and with standard interferon plus ribavirin and amantadine (Group B). Percentages of patients negative for HCV-RNA during and after therapy with peginterferon plus ribavirin and amantadine (black bar) or standard interferon plus ribavirin and amantadine (white bar) are shown. Significance between the two treatments is shown at each time interval.

20.7%, $P = 0.036$). The reasons for discontinuation are reported in Table 4. Dosage reduction, reported in 30 patients (17 treated with PegIFN and 13 with standard IFN), was related to hematological alterations in 12 of the patients who received pegylated interferon and in 7 of those treated with standard interferon. Dermatological manifestations (mainly reported in patients treated with pegylated interferon) and cephalgia were the causes of dosage reduction in the remaining patients. Adherence

Table 3 Sustained virologic response in patients treated with Peg IFN or standard IFN according to the baseline characteristics of patients

Characteristic	PegIFN + RBV + AMA		IFN + RBV + AMA		P
Genotype 1	21/82	26%	11/65	17%	NS
Non 1	13/24	54%	5/17	29%	NS
Age ≤ 40 yr	11/23	48%	2/19	11%	0.017
> 40 yr	23/83	28%	14/63	22%	NS
BMI ≤ 25 kg/m ²	22/60	37%	10/47	21%	NS
> 25 kg/m ²	8/33	24%	4/28	14%	NS
Stage ≤ 3	27/71	38%	10/56	18%	0.018
> 3	6/30	20%	6/26	23%	NS
Grade ≤ 10	28/94	30%	14/73	19%	NS
> 10	6/9	67%	2/6	33%	NS
ALT ≤ 120 UI/L	17/64	27%	5/43	12%	NS
> 120 UI/L	15/35	43%	9/31	29%	NS
HCV-RNA ≤ 2 × 10 ⁶ copies/mL	15/41	37%	8/33	24%	NS
> 2 × 10 ⁶ copies/mL	6/13	46%	1/15	7%	0.029

NS: Not significant.

lower than 70% in the first 24 wk of therapy was observed in 8/106 (7.5%) patients in treatment A and in 8/82 (10%) in treatment B. No significant difference in the rate of response was observed between patients who did or did not reduce therapy.

DISCUSSION

This is the first randomized study to compare the efficacy of two triple therapy regimens-recombinant interferon α -2a either standard or pegylated, in combination with ribavirin and amantadine-in a well-selected series of patients nonresponsive to previous treatment with interferon and ribavirin. Our data demonstrate that after 12 mo of treatment, a sustained virologic response is obtained in a surprisingly high number of these difficult-to-treat patients. Better results in terms of sustained response (32% *vs* 19% of SVR) were observed in patients treated by pegylated interferon than in those treated by a high daily dose of standard interferon. Baseline elevated ALT level and undetectable serum HCV RNA at wk 12 of therapy were the independent predictors of sustained response, irrespective of the interferon used (pegylated interferon or high dose standard interferon). As a whole, triple therapy was well tolerated and safe, with a higher frequency of therapy discontinuation in patients treated by standard interferon than in those treated by pegylated interferon.

The overall rate of SVR observed in the present study of nonresponders to combination therapy (standard interferon plus ribavirin), most of whom had been already submitted to several treatments, is higher than that generally observed in previously reported studies. The results are even more impressive when considering patients treated by pegylated interferon. In particular, patients with high viral load seemed to respond better when treated with pegylated interferon, even if the small number of cases in which basal viral load was available does not allow definite conclusions to be drawn. Only a few studies have been carried out to assess the efficacy of retreatment in patients

Table 4 Reasons for treatment discontinuation

	PegIFN + RBV + AMA (n = 106)	IFN + RBV + AMA (n = 82)
Treatment discontinuation for any Reason ¹	10 (9.4%)	17 (20.7%)
Study drug noncompliance	2	11
Demyelinating neuropathy	1	
Depression	1	1
Erythema		1
Pulmonary fibrosis	1	
Hyperthyroidism	1	
Hyperthyroidism & Insomnia	1	
Increased ALT		1
Leukopenia	1	3
Dyspnoea, Cough, Pruritus	1	
Unknown reason	1	

¹ Fisher's exact test: *P* = 0.036.

nonresponsive to combination therapy and most of them included small series of patients and did not disaggregate relapsers from true nonresponders^[24,25,27]. Schiffmann *et al*^[14] reported an overall SVR rate of 18% after retreatment for 48 wk with PegIFN plus RBV, and even lower rates were observed in patients with unfavourable predictors such as genotype 1 and/or high viral load. Teuber *et al*^[28] reported a 22% SVR in previous nonresponders either to interferon or combination therapy when retreated with triple therapy including amantadine, however, the SVR rate dropped down to 12% in those patients who had previously been given two or more courses of antiviral treatment. Typically, SVR rates in nonresponders to combination therapy retreated with triple therapy have averaged approximately 12%^[29-32], although in a recent placebo-controlled study the SVR with amantadine triple therapy was 24%^[33], while in a small group of genotype 1, nonresponsive patients without cirrhosis, a SVR of 42% was reported^[35].

We cannot exclude that a selection bias may partially explain the high rate of response found in our study. Although the main clinical and virologic characteristics (age, prevalence of genotype 1) of our patients are those usually observed in nonresponders to combination therapy, in our series the prevalence of cirrhosis was only 16% and the majority of patients had a fibrosis stage lower than 3 according to the Ishak score, differing from Schiffman's^[14] study which included only patients with bridging fibrosis or cirrhosis. However, we observed a high SVR (30%) in patients with cirrhosis (stage 5 and 6) by the Ishak score and no significant difference in sustained response between patients with or without cirrhosis. We are therefore more prone to think that the higher antiviral efficacy found in our study is more likely linked to the addition of amantadine to combination therapy. In keeping with our results Maynard *et al*^[33] demonstrated that 24% of non responders treated by triple therapy achieved an SVR of 16% of those treated by double therapy. The mechanism by which amantadine enhances the antiviral effect of interferon and ribavirin is unclear and remains largely speculative. It is well known that amantadine may act as an antiviral drug and its synergistic action in combination with IFN and RBV may function through the modulation of the host immune-response^[36]. Although the mechanism

underlying the potential synergism between these drugs is not clear, our results suggest that triple therapy, in particular when PegIFN is used, has to be considered as a valid option in nonresponders to combination therapy since it allows a sustained viral clearance in about one third of otherwise nonresponsive patients.

In keeping with the results obtained in treatment-naïve patients and in relapsers^[1-4], PegIFN achieved a better response than standard interferon even though the latter was administered at a high daily dosage. Although the difference in SVR observed between the two treatment groups did not reach a statistical difference ($P = 0.057$), our results strongly suggest that in nonresponders, pegylated interferon should represent the first choice interferon in retreatment strategies. Our suggestion is further strengthened by the evidence of a lower incidence of therapy discontinuation in patients treated with PegIFN than in those treated with high dose standard interferon.

Baseline ALT value and undetectable serum HCV RNA at wk 12 of therapy were the variables independently associated with sustained response. Unexpectedly, HCV genotype did not emerge as an independent predictor of response by univariate analysis. In fact, in the present series, although genotype 1 patients responded less well than those with other genotypes, the difference did not maintain the significance at multivariate analysis. One possible explanation of this discrepancy is that the previous treatments may have selected patients in whom several and possibly new mechanisms of therapeutic resistance have emerged over time. In the present series the SVR in patients with genotype 1 was 22% (26% and 17% in those treated with PegIFN or standard IFN, respectively), a rate that is markedly higher than reported in other studies^[14,37]. Interestingly, in treatment-naïve patients infected with genotype 1 the addition of amantadine to interferon therapy also led to an increased rate of SVR^[38]. Although the problem remains of whether or not the retreatment of genotype 1 nonresponders is justified in terms of cost-efficacy^[39], at the moment triple therapy including amantadine should be regarded as the most useful regimen to be proposed for these patients.

In the present study the addition of amantadine did not negatively affect the safety profile of combination therapy. In fact, triple therapy was found to be safe and well tolerated in the large majority of patients and the frequency and type of side effects did not differ from that observed in other series of nonresponders treated with standard or pegylated interferon plus ribavirin^[1,2,40,41]. The higher rate of treatment withdrawal in patients treated with high dose standard recombinant interferon than in those receiving pegylated interferon parallels the results observed in treatment-naïve patients receiving pegylated interferon when compared to high dose induction therapy^[42]. Interestingly, in our study, only two patients experienced depression severe enough to require treatment discontinuation. It is possible that the addition of amantadine, which is known to possess antidepressant properties^[43,44], may have decreased the overall incidence of depression, which is often the cause of premature therapy withdrawal. This is another relevant aspect that, in association with the low cost of amantadine, supports the

use of triple therapy in nonresponsive patients.

Although our study was not specifically designed to assess the predictive value of virologic response, some information can be drawn. Undetectable serum HCV RNA at wk 12 of therapy was the strongest independent predictor of SVR, in fact 50% of the patients who were HCV-RNA negative after 12 wk of treatment and 10% of those who were still positive, became sustained responders. In particular, the predictive negative value (90%) observed in our series at week 12 is slightly lower than that usually observed in treatment-naïve patients receiving combination therapy^[45], suggesting that it may be more useful to wait until wk 24 before deciding whether to withdraw therapy. These findings further confirm that in this group of difficult-to-treat patients the variables and prognostic factors usually associated with response cannot be employed.

In conclusion, the present multicenter randomized study demonstrated that triple therapy, in particular employing pegylated- α -2a interferon plus ribavirin and amantadine, is an effective therapeutic option for patients nonresponsive to previous treatment with interferon plus ribavirin and can rescue at least one-fourth of these patients to sustained viral clearance. Although many questions remain regarding the role of amantadine in the treatment of HCV chronic hepatitis, these results, taken together with data from recent studies, provide encouragement for retreatment of nonresponders to combination therapy with triple therapy. Although our results need to be confirmed further in placebo-controlled studies, they seem to be particularly interesting when taking into account that addition of amantadine is low cost and safe and could offer hope to patients for whom no alternative therapies are available.

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Detection of YMDD mutation using mutant-specific primers in chronic hepatitis B patients before and after lamivudine treatment

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Abstract

AIM: To develop a PCR assay using mutant-specific primers to detect mutation of tyrosine-methionine-aspartate-aspartate (YMDD) motif of HBV to tyrosine-valine-aspartate-aspartate (YVDD) or tyrosine-isoleucine-aspartate-aspartate (YIDD).

METHODS: Cloned wild-type and mutant HBV sequences were used as templates to test the sensitivity and specificity of the assay. A variety of primer construction, primer concentration, dNTP concentration, and annealing temperature of primers were systematically examined. Pair primers specific to rtL180M and rtM204V were selected for YVDD detection. Primer specific to rtM204I with an additional 3'-penultimate base mismatched to both the mutant and wild-type sequence was selected for YIDD detection. We applied this assay to study YMDD mutants in 28 chronic hepatitis B patients before and after lamivudine treatment.

RESULTS: We could detect as little as 0.001%-0.00001% of mutant viruses coexisting in 10^8 - 10^9 copies of wild-type HBV using this assay. YMDD mutants were detected in 8 of 12 HBeAg-positive patients and 8 of 16 HBeAg-negative patients before lamivudine treatment. After treatment, two more patients in HBeAg-positive patients and seven more patients in HBeAg-negative patients developed YMDD mutations.

CONCLUSION: We developed a highly sensitive and specific assay for detecting YMDD mutants. This assay can be applied to monitor chronic hepatitis B patients before and during lamivudine treatment.

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Key words: Hepatitis B virus; Lamivudine; Tyrosine-

INTRODUCTION

Hepatitis B virus (HBV) is one of the most common infectious diseases in the world. More than 300 million people worldwide are estimated to have chronic HBV infection. Ten percent of these patients will die as a direct consequence of persistent viral infection^[1]. Nucleoside analogue therapy allows safe, long-term suppression of HBV and is a major milestone in the treatment of chronic hepatitis B. Lamivudine, the first of these agents approved worldwide, effectively suppresses viral replication, reduces disease activity, improves liver histology, and delays clinical progression^[2-4]. However, the development of lamivudine resistant mutations occurs in 14%-32% of patients after 1 year of therapy^[5,6]. The longer the treatment is continued, the more frequently resistance is seen (65% at 5 years)^[7]. Lamivudine-resistant HBV is associated with mutations of the YMDD motif in the polymerase gene. The key mutations are the substitutions of methionine at the rtM204 (domain C) to either isoleucine (rtM204I, YIDD variant) or valine (rtM204V, YVDD variant)^[8,9]. The rtM204V variant is almost always accompanied by an additional rtL180M mutation in the domain B^[10,11].

Several assays, including DNA sequencing^[12], restriction fragment length polymorphism (RFLP)^[13,14], peptide nucleic acid (PNA) mediated PCR clamping^[15], line probe assay^[16,17], and oligonucleotide microarray^[18,19], have been developed to detect YMDD mutations. The best sensitivity achieved so far was 10^4 copies of variant in the mixture of 10^9 copies of the wild-type virus and 10^1 in 10^5 of wild-type^[15].

In this study, we developed a simple but highly sensitive and specific assay using mutant-specific primers to detect lamivudine-resistant mutations. We applied this method to detect YMDD mutants in chronic hepatitis B patients before and after lamivudine treatment.

MATERIALS AND METHODS

Clinical samples and method for extraction of HBV DNA

Serum samples were collected from 28 chronic hepatitis B patients who had histologically confirmed cirrhosis and had received lamivudine treatment for 12 to 48 mo. Informed consent was obtained from all patients according to the ethical guidelines of the 1975 Declaration of Helsinki. HBV DNA was extracted from serum using a DNA extraction kit (Qiagen, Hilden, Germany).

Subcloning of the HBV gene

Sera with wide-type HBV DNA, YVDD or YIDD mutants were amplified by PCR with a primer set (sense primer: 5'-GATGTGTCTGCGGCGTTTAA-3', antisense primer: 5'-CAGCAAAGCCCCAAAGACCCAC-3'). The obtained PCR products (625bp, containing site codons rt180 and rt204) were then cloned into pOSI-T vector (GeneMark, Taichung, Taiwan) using the standard method.

Detection of YMDD mutation using PCR with mutant-specific primers

We used cloned YVDD, YIDD and wild-type HBV genomes as templates to determine the optimal conditions of mutant-specific PCR. A variety of primer construction (Table 1), primer concentration, dNTP concentration and annealing temperature of primers were systematically examined (data not shown). The primers finally selected were listed with boldface in Table 1. The single-step PCR for YVDD detection was carried out with the primers YVDD669 and YVDD758R in a 25 μ L reaction volume containing 10 mmol/L Tris HCl, pH 8.3, 50 mmol/L KCl, 2.0 mmol/L MgCl₂, 0.03 mmol/L of each dNTP, 2.5 pmol of each primer, 1.5 units of Taq polymerase (Viogene, Taipei, Taiwan). The PCR condition included initial denaturation for 3 min at 94°C, 48 cycles of amplification with denaturation at 94°C for 30 s, primer annealing at 57°C for 30 s, extension of primer at 72°C for 25 s, and final extension at 72°C for 1 min. The nest-PCR was performed with HBV376 and HBV1021R as outer primers. The first PCR was conducted in a 20 μ L reaction volume with 0.0375 mmol/L dNTP and 1 pmol of each outer primer. The PCR condition was initial denaturation for 3 min at 94°C, 30 cycles of amplification with denaturation at 94°C for 30 s, primer annealing at 53°C for 30 s, extension of primer at 72°C for 50 s, and final extension at 72°C for 2 min. One microliter of the first reaction was used for the second PCR. The second PCR condition was the same as the single-step PCR except the annealing temperature was 55°C. The single-step PCR for YIDD detection was carried out with primers HBV379 and YIDDR2T in the same reaction condition as for YVDD detection except the annealing temperature was 59°C. The first PCR of the nest-PCR for YIDD detection was performed in the same condition as for YVDD detection. One microliter of the first reaction was used for the second PCR. The second PCR was carried out in a 25 μ L reaction volume with 0.03 mmol/L of each dNTP, 2.5 pmol of each primer (HBV485 and YIDDR2T) and 0.1U of Perfect Match PCR enhancer (Stratagene, La Jolla, California, USA), 1.5 units of Taq polymerase. The reaction condition was initial one cycle of

Table 1 DNA sequence of the primers tested for detection of YMDD mutation

¹ Primers	² DNA sequence	Nucleotide
YVDD758	5'-CACTGTTTGGCTTTCAGTTATG (A)	737-758 Sense
YVDD758R	5'-CCCAAAACCACATCATCCAC (T)	758-739 Antisense
YVDD669	5'-GGGCCTCAGTCGTTTCTCA (T)	650-669 Sense
YIDD	5'-CTGTTTGGCTTTCAGTTATATT (G)	739-760 Sense
YIDD2G	5'-GTTTGGCTTTCAGTTATAGT (TG)	741-760 Sense
YIDD2A	5'-GTTTGGCTTTCAGTTATAAT (TG)	741-760 Sense
YIDD2C	5'-GTTTGGCTTTCAGTTATACT (TG)	741-760 Sense
YIDDR	5'-CCCCCAAACCACATCATCA (C)	760-745 Antisense
YIDDR2T	5'-CCCCCAAACCACATCATTA (CC)	760-745 Antisense
YIDDR2A	5'-CCCCCAAACCACATCATAA (CC)	760-745 Antisense
YIDDR2G	5'-CCCCCAAACCACATCATGA (CC)	760-745 Antisense
HBV376	5'-GATGTGTCTGCGGCGTTTAA	376-395 Sense
HBV485	5'-CCAGGAACATCAACCACCAG	485-504 Sense
HBV1021R	5'-CAGCAAAGCCCCAAAGACCCAC	1021-1000 Antisense

¹ The primers finally selected are indicated in boldface; ² The engineered mismatched nucleotides are underlined; the wild-type nucleotides are shown in parenthesis.

94°C for 5 min, 62°C for 5 min and 72°C for 30 s, 48 cycles of amplification with denaturation at 94°C for 30 s, primer annealing at 62°C for 30 s, extension of primer at 72°C for 30 s, and final extension at 72°C for 1 min. The PCR products were subjected to electrophoresis in a 3% agarose gel.

HBV DNA quantification and Sequencing

HBV DNA quantification was performed using the Lightcycler real-time PCR assay^[20]. PCR amplification of the HBV genome was carried out using the same primers as subcloning of HBV DNA described above. PCR-amplified HBV DNA was purified and sequenced using specific sequencing primers and a commercial sequencing Kit (ABI Prism Dye Terminator Cycle Sequencing, Perkin Elmer, Warrington, UK). The sequencing reactions were analyzed on an automatic DNA sequencer.

RESULTS

We assessed the sensitivity and specificity of this method using mutant type and wild-type HBV plasmid. For single-step PCR, the detection limit was 10⁴ copies for YVDD variant or 8 × 10⁴ copies for YIDD variant (data not shown). For nest-PCR, the detection limit was up to 10¹ copies for both variants (Figure 1A, B). Wild-type HBV, even up to 10¹⁰ copies, was not detected using these mutant-specific primers for both single-step PCR (data not shown) and nest-PCR (Figure 1A, B). We then tested the mixture of wild-type and mutant HBV using nest-PCR. Various copies of wild-type plasmid (10⁷-10¹⁰ copies) were mixed with YVDD variant at the indicated concentrations (10¹-10⁴ copies). In the presence of 10⁸ wild-type sequences, a total of 10¹ copies of both variants could be detected (Figure 1A, B). A total of 10⁴ copies of YVDD variants or 10³ copies of YIDD variants were detected in the mixture of 10⁹ copies of the wild-type sequences (Figure 1A, B). Thus, the detection limit was

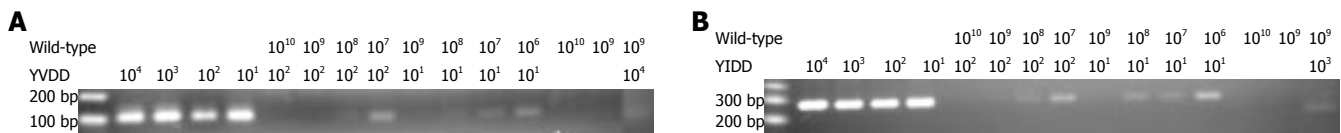


Figure 1 Sensitivity and specificity of the YMDD mutant detection by mutant-specific PCR. **A:** Various copies of wild-type sequence, YVDD mutant sequence, and mix of both sequences at the indicated concentrations. The left most lane contains molecular weight marker in base pair unit. The detection limit was 10¹ copies of YVDD mutant in 10⁸ copies of wild-type HBV or 10⁴ copies of YVDD mutant in 10⁹ copies of wild-type HBV; **B:** Various copies of wild-type sequence, YIDD mutant sequence, and mix of both sequences at the indicated concentrations. The left most lane contains molecular weight marker in base pair unit. The detection limit was 10¹ copies of YIDD mutant in 10⁸ copies of wild-type HBV or 10³ copies of YIDD mutant in 10⁹ copies of wild-type HBV.

Table 2 Clinical Characteristics of 28 patients with chronic hepatitis B before and after long-term treatment of lamivudine

No.	Before treatment				After treatment				
	AST/ALT (IU/L)	HBeAg/anti-HBe	¹ HBV-DNA	² Nest-PCR	AST/ALT (IU/L)	HBeAg/anti-HBe	¹ HBV-DNA	Nest-PCR	³ Sequencing
1	61/62	-/+	39.93	-	29/16	-/+	0.25	V	V
2	31/43	+/-	3574.29	V	29/54	+/-	4400.65	M	V
3	85/86	-/+	348.66	-	20/11	-/+	0.20	-	W
4	45/90	-/+	8.50	-	23/22	-/+	0.03	-	W
5	74/75	-/+	256.99	I	63/38	-/+	0.01	I	I
6	82/163	-/+	1596.12	-	38/64	-/+	3356.38	V	V
7	37/43	+/-	13.07	-	25/39	+/-	0.26	-	W
8	26/62	-/+	49.16	-	24/40	-/+	0.03	-	W
9	82/102	-/+	3313.93	-	29/17	-/+	0.60	V	W
10	40/59	+/-	546.76	I	24/28	-/+	0.11	M	I
11	117/232	+/-	49.50	I	26/27	+/-	346.96	M	I
12	46/61	-/+	47.40	-	24/32	-/+	0.06	V	V
13	72/107	-/+	5.37	M	66/92	-/+	2389.09	M	V
14	155/159	+/-	2325.13	I	22/14	+/-	0.10	M	W
15	65/54	-/+	1461.13	-	42/29	-/+	0.12	V	W
16	133/157	+/-	7943.81	I	73/64	+/+	2.05	I	I
17	107/146	-/+	23.13	M	151/232	-/+	59.94	M	V
18	35/48	+/-	1666.59	-	22/21	+/-	0.15	-	W
19	247/183	+/-	9336.17	I	27/14	+/-	0.01	I	W
20	45/56	-/+	219.58	-	28/38	-/+	0.03	-	W
21	109/186	-/+	61.27	-	30/48	-/+	2.91	M	I
22	173/335	+/-	11.98	-	29/31	+/-	0.03	M	V
23	51/81	-/+	1403.11	I	28/42	-/+	303.09	I	I
24	43/68	+/-	365.07	-	22/27	+/-	1.28	V	V
25	45/57	+/-	7530.63	I	30/25	+/-	6228.83	I	I
26	130/286	-/+	126.67	-	21/32	-/+	1.46	I	I
27	64/91	+/-	4700.63	I	52/73	+/-	6.12	I	I
28	88/164	-/+	0.38	-	30/38	-/+	0.07	-	W

¹ Unit (10⁵ copies/mL); ² M: YVDD + YIDD, V: YVDD, I: YIDD, -: YVDD and YIDD not detectable; ³ W: wild-type, V: YVDD, I: YIDD.

0.0001%-0.00001%.

We then applied this method to 28 chronic hepatitis B patients who had been treated with lamivudine for 12 to 48 mo (Table 2). Before treatment, there were 12 patients with positive HBeAg and 16 patients with negative HBeAg and positive anti-HBe. Among 12 HBeAg-positive patients, four were free of YMDD mutant, seven with YIDD mutant, and one with YVDD mutant. One developed HBeAg sero-conversion after treatment. Among 4 patients with initial negative YMDD mutant, two developed mixed mutation, and two remained free from YMDD mutation. Among 7 patients with initial YIDD mutant, three developed mixed mutations, and four remained with

YIDD mutation. The patient with initial YVDD mutant developed mixed mutation. Among 16 HBeAg-negative patients, before treatment, twelve were free of YMDD mutation, two with YIDD mutation, and two with mixed mutation. Among 12 patients with initial negative YMDD mutant, five developed YVDD mutation, one developed YIDD mutation, one developed mixed mutation, and five remained free of YMDD mutation after treatment. The other four patients, two with YIDD mutant, two with mixed mutation, remained with the same mutation. Conventional sequencing of post-treatment sera showed 11 cases of wild-type HBV, 8 cases of YVDD mutation, and 9 cases of YIDD mutation. Among 11 cases of wild-

type HBV, eight were wild-type, two were YVDD, one was YIDD, and one was mixed mutation by nest-PCR. Among 8 cases of YVDD mutation, four were YVDD and four were mixed mutation by nest-PCR. Among 9 cases of YIDD mutation, six were YIDD and three were mixed mutation by nest-PCR (Table 2). Overall, mutant-specific PCR agreed with sequencing on YMDD mutation detection, but was more sensitive in detecting additional mutations.

DISCUSSION

Taq DNA polymerases extend mismatches much less efficiently than correct matches. Depending on the nature of the mismatch composition, the elongation efficiency of the mismatched duplex can vary from 0.0001% to 0.1% of the elongation efficiency of the perfectly matched duplex^[21]. Reduced dNTP and primer concentration can increase the specificity of mutant-specific PCR^[22]. We have lowered 2-4 fold the dNTP and primer concentration of conventional PCR and compensated for the reduced efficiency by increasing amplification cycles. With this modified PCR condition, we found that in the presence of 10^6 to 10^7 copies of wild-type HBV DNA, a single 3'-end mismatch primer still showed a false positive result (data not shown). For the YVDD mutation, rtL180M mutation is almost always accompanied by an additional the rtM204V mutation^[10,11]. We therefore selected pair primers with 3'-end specific to rtL180M and rtM204V to detect YVDD mutation. For YIDD mutation detection, we designed primers containing a mutant-specific 3'-terminal base and a 3'-penultimate base mismatched to both the mutant and wild-type sequence. We checked 3 forward (YIDD2G, YIDD2A, and YIDD2C) and 3 reverse (YIDDR2T, YIDDR2A, and YIDDR2C) primer constructions. The YIDDR2T primer had the greatest specificity and sensitivity in detecting YIDD mutation. Besides, we add Perfect Match PCR enhancer to increase the specificity of the assay^[23]. The present assay had a detection limit of 0.0001%-0.00001%, which was better than that of PNA mediated PCR clamping (0.001%-0.0001%)^[15] and that of RFLP assay (1%-10%)^[13]. However, the latter two methods have the advantage of detecting both wild-type and mutant HBV simultaneously in one reaction. The choice of assay should depend on the context of the application.

Hepatitis flare can occur during lamivudine treatment because of the emergence of YMDD mutants. The serum titer of the mutant viruses during hepatitis flare is well above 10^5 copies/mL^[24]. The single-step PCR in this study only detected YMDD mutation at copy number greater than 10^4 , and can be applied in this clinical context. In fact, we have applied single-step PCR to check two cases of hepatitis flare during lamivudine treatment. It correlated well with HBV DNA quantification and sequencing (data not shown).

Because HBV employs reverse transcription to copy its genome, mutant viral genomes are found frequently. Under conditions of high levels of viral replication, it is likely that HBV mutations conferring resistance to lamivudine may pre-exist. Indeed, pre-existing YMDD mutants have been demonstrated in some lamivudine-naïve asymptomatic

HBV carriers^[25] or chronic hepatitis B patients^[15]. The patients with pre-existing YMDD mutants were all positive for anti-HBe antibody^[15,25]. In this study, twelve patients were shown to have pre-existing YMDD mutants, and eight of them were HBeAg-positive. Seven cases with pre-existing mutation had a serum HBV DNA level greater than 10^8 copies/mL. However there were also cases with a high level of HBV DNA but without a pre-existing mutation. We suspect that with more sensitive detecting methods, more cases of pre-existing mutation will be demonstrated. The patients with a pre-existing mutation had persistent mutation or developed mixed mutation during treatment, but only five of them had a poor virological response. Whether patients with a pre-existing lamivudine-resistant mutation should be treated with other nucleoside analogues requires further prospective studies.

In conclusion, we have developed a sensitive and specific method using mutant-specific primers to detect the YMDD mutation. This PCR assay might be an effective procedure for monitoring chronic hepatitis B patients treated with lamivudine or screening them before starting this anti-viral therapy.

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H pylori

***H pylori* receptor MHC class II contributes to the dynamic gastric epithelial apoptotic response**

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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 a marked 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 results presented here demonstrate that the ability of MHC class II to modulate gastric epithelial apoptosis is at least partially dependent on its crosslinking. Furthermore, while previous research has demonstrated that MHC class II signaling can be pro-apoptotic during extended ligation, we have shown that the crosslinking of this molecule has anti-apoptotic ef-

fects 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 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*; MHC class II; Gastric epithelial cell; Apoptosis

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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, the methods by which 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 which 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 the *H pylori* niche within the gastric mucosa. Thus, mechanisms by which this 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^[6,7]. 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. This, combined with our previous data demonstrating the role of MHC class II in *H pylori* binding to gastric epithelial cells (GEC), suggests 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. We hypothesize that MHC class II has differential, and possibly opposing, effects on epithelial cell apoptosis during *H pylori* infection depending on several factors, including crosslinking *vs* simple ligation, the duration of MHC class II interaction with the bacteria, and MHC class II interaction with other cell surface signaling molecules. More specifically, we suspect that complex surface crosslinking of MHC class II will have a distinctly different effect on the recruitment and activation downstream accessory and effector molecules, such as FADD and caspases, when compared to non-crosslinking ligation.

MATERIALS AND METHODS

Cell culture

The human gastric epithelial cell line N87 was obtained from ATCC and cultured in RPMI containing 10% fetal calf serum and supplemented with glutamine.

Bacterial culture

H pylori cag+ clinical isolate LC-11^[8] was grown on a blood agar base (Becton Dickinson) at 37°C under microaerobic conditions and harvested into Brucella broth containing 10% 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 ($1\text{ A} = 2 \times 10^8\text{ cfu/mL}$) (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 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 2 h incubation at 37°C, the cleaved substrate product resulting from the action of activated caspases was quantitated using a fluorimeter ($\gamma = 521\text{ nm}$).

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 U/mL IFN γ for 48 h to induce the upregulation of

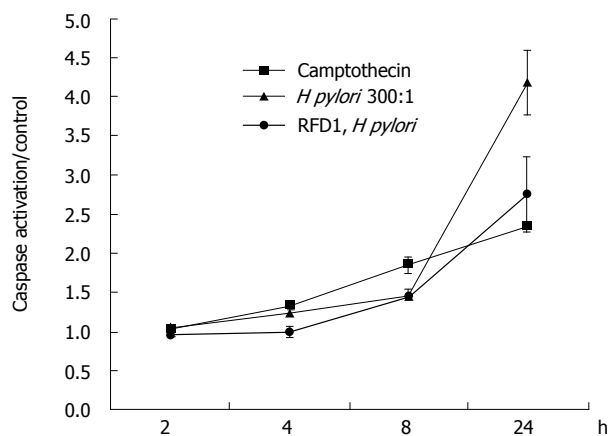


Figure 1 Total caspase activation was detected using a colorimetric assay. Values are expressed as the means of the treatments/control (untreated) samples.

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 mol/L) was used as a positive control for caspase activation and an isotype antibody, which had no effect on the cells. 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 to the activated form of caspase 3. Two experimental groups and one untreated, control group were used. (All groups were pre-treated with IFN γ to upregulate surface MHC class II). The first experimental group consisted of cells treated with a monoclonal IgG antibody cocktail specific to MHC class II. The purpose of this group is to mimic simple bacterial ligation to MHC class II. The second experimental group contained cells treated with biotinylated anti-MHC class II antibodies plus streptavidin. The purpose of this treatment group is to mimic complex MHC class II crosslinking by *H. pylori*. The untreated group was not exposed to *H. pylori* or antibodies. At 4 and 8 h treatment of 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 activation 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 just 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

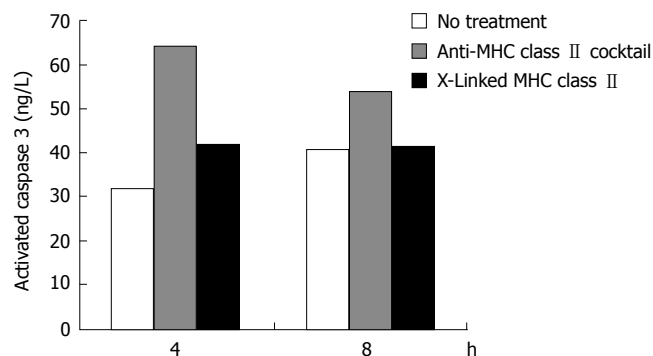


Figure 2 The effects of ligating versus crosslinking N87 cell surface MHC class II molecules on caspase 3 activation. Using the apoptosis cytometric bead array kit, beads exposed to the samples were run through a flow cytometer to obtain MFI data. These results were normalized to activated caspase 3 standards to give values expressed as ng/L of activated caspase 3.

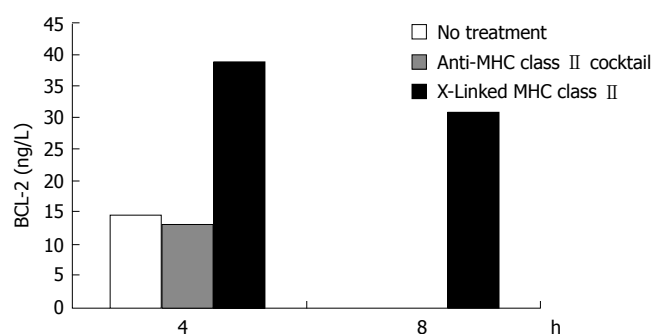


Figure 3 The effects of ligating versus crosslinking N87 cell surface MHC class II molecules on BCL-2 expression. Using the apoptosis cytometric bead array kit, beads exposed to the samples were run through a flow cytometer to obtain MFI data. These results were normalized to BCL-2 standards to give values expressed as ng/L of BCL-2.

was measured simultaneously with caspase 3 activation. Just 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 detectible BCL-2 (Figure 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 U/mL 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

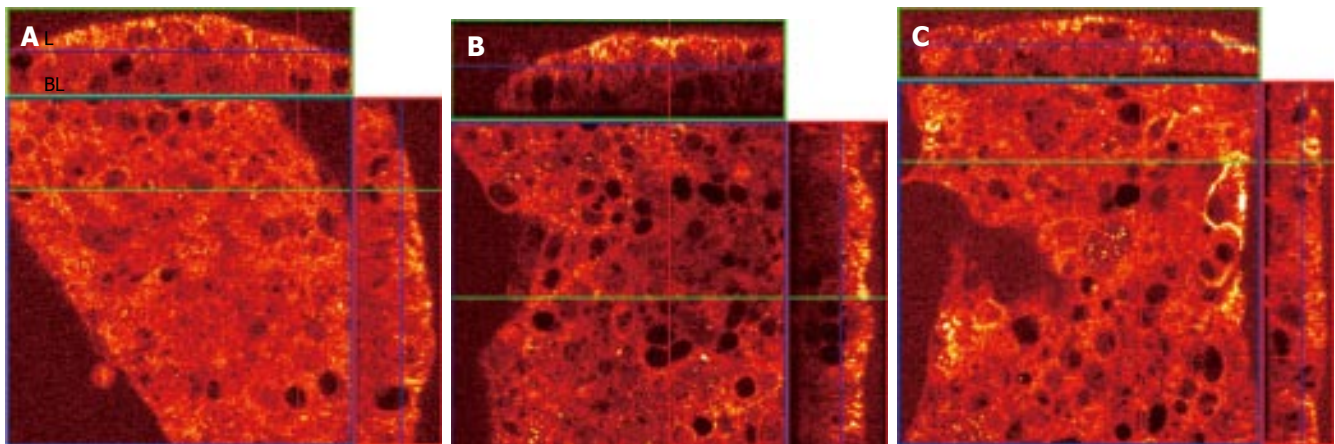


Figure 4 A: Viewing the X-Z panels, the FADD (stained red, with higher densities staining yellow) in untreated (control) cells can be seen distributed equally throughout the cytoplasm. "L" represents the apical (luminal) surface and "BL" represents the basolateral surface of the N87 monolayer; B: Labeled 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 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 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 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 concluding that 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 that MHC class II not only binds *H pylori*, but also can initiate signals affecting apoptotic pathways, suggests an important role for this molecule in influencing *H pylori* pathogenesis. The literature offers conflicting reports on the apoptotic versus 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. These findings become more logical in hindsight when one considers a key characteristic of the MHC class II heterodimer: its cytoplasmic tail is very short, consisting of 12-15 amino acids. Just as a T cell receptor requires crosslinking and association with other surface molecules to recruit accessory proteins and effect downstream events, we suspect that the short tail of MHC class II leads to the necessity for crosslinking and interaction with other surface molecules to alter apoptotic pathways. Recent studies by our group have led to the discovery that *H pylori* also binds to the MHC class II-associated invariant chain (Ii)^[21]. Because the cytoplasmic tail of Ii is significantly longer, and potentially more potent as a functional recruiter of transduction molecules, we envision a scenario in which simple ligation of *H pylori* to either MHC class II or Ii might induce a pro-apoptotic effect via the cytoplasmic tail of Ii. This pro-apoptotic scenario would functionally oppose the result of a more extensive bacterial interaction with the epithelium, leading

to crosslinked MHC class II with the resulting increase in BCL-2 and suppression of FADD recruitment, leading to a reduction of apoptosis. The method by which the crosslinking of MHC class II inhibits CD95-mediated apoptosis is, at this point, speculation. However, we hypothesize that one explanation, focusing on a presumed intimate association between MHC class II and Fas on the epithelial surface, is that crosslinked (but not ligated) MHC class II sterically inhibits the trimerization, and thus activation, of surface Fas molecules.

Our findings that MHC class II crosslinking inhibits *H pylori*-induced caspase activation, that ligation versus 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 not only because of the need to understand the molecules responsible for bacterial adhesion, but also 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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First-line eradication of *H pylori* infection in Europe: A meta-analysis based on congress abstracts, 1997-2004

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Abstract

AIM: To meta-analyse the European abstracts presented between 1997-2004 at the European *H pylori* Study Group, United European Gastroenterology Week meetings and World Congresses of Gastroenterology.

METHODS: The abstracts of randomized/controlled prospective studies were classified into groups based on first-line eradication schedules. The quality of the abstracts was checked by a validated score system. The pooled eradication rates (PER) and combined odds ratios (OR) were calculated and compared with the published meta-analyses.

RESULTS: The PER of proton pump inhibitor-based (PPI) one week triple therapies was 81.4% (confidence interval, 95% CI: 78.5-84.5). Ranitidine bismuth citrate-based (RBC) triple regimens have an efficiency rate of 78.5% (95% CI: 70.5%-84.3%) ($P = 0.28$ vs PPI). The OR for PPI effect vs RBC regimens was 1.1 (95% CI: 0.92-1.30). H_2 receptor antagonist-based triple therapies achieved 64.1% (95% CI: 52.6-75.6) ($P = 0.02 < 0.05$ vs PPI), the OR vs PPI regimens was 1.55 (95% CI: 0.72-3.78). PPI-based double combinations were less efficient than triple regimens (PER: 55.0%, OR: 4.90, 95% CI: 2.36-9.70). Quadruple regimens were successful in 82.6% (95% CI: 76.0-89.7), the OR vs triple therapies was 0.80 (0.62-1.03). Clarithromycin + amoxicillin or nitroimidazole combinations were efficient in 80.5% (95% CI: 77.2-84.2) and 83.8% (95% CI: 81.7-85.9), respectively. Amoxicillin + nitroimidazole therapies eradicated the infection in 73.5% (66.6-78.5) ($P = 0.01 < 0.05$ vs clarithromycin-based regimens).

CONCLUSION: PPI/RBC-based triple therapies achieved comparable results with the meta-analyses. H_2 -receptor antagonists and PPI-based double combinations were less efficient. Triple and quadruple regimens were

equally effective. Clarithromycin + either amoxicillin or nitroimidazole containing regimens were more effective than amoxicillin + nitroimidazole combinations. High quality congress abstracts constitutes a valuable pool of data which is suitable for meta-analytical workup.

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Key words: Antimicrobials; Eradication; *H pylori*; Proton pump Inhibitors; Ranitidine bismuth citrate

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<http://www.wjgnet.com/1007-9327/12/5311.asp>

INTRODUCTION

Numerous treatment regimens have been tested for their efficacy in curing *H pylori* infection worldwide^[1-3]. Meta-analysis has become a frequently used method for resolving such uncertainties and obtaining sound data in evidence-based medicine. The purpose of a meta-analysis is to statistically combine the results of similar trials and such studies are aimed at improving the estimation of treatment effects and minimizing the potential biases of such estimations. Following the increased rate of randomized controlled trials (RCT) published, the importance of meta-analyses has risen and its methodology has become more and more sophisticated. This is particularly true for *H pylori* eradication where we see an exponential rise of studies published worldwide. As a part of the evidence-based medicine, most of the meta-analyses only addressed the RCTs. However, it was rapidly realized, that RCTs do not come close to covering the complete range of studies and therefore, the inclusion of studies representing lower levels of evidence or congress abstracts was also accepted in some of the studies^[2,3]. Score systems for assessing the quality of meta-analyses, RCTs^[4,5] and abstracts^[6] have also been worked out. Participation at scientific meetings represents a popular opportunity to disseminate results which, because of linguistic barriers would hardly be published in peer-reviewed journals.

The aim of our study was the meta-analysis of the European abstracts dealing with the first-line treatment of the

H. pylori infection, presented at the European *H. pylori* Study Group (EHPSG), United European Gastroenterology Week (UEGW) and World Congress of Gastroenterology (WCOG) meetings held between 1997-2004. We thus investigated an unexplored area which was only in part covered by meta-analyses published so far.

MATERIALS AND METHODS

Data collection

Abstracts presented at the EHPSG, UEGW and WCOG meetings between 1997-2004 were reviewed independently by the authors. These meetings were chosen because acceptance of the papers is decided after a peer-review process similar to submitting to journals. The randomized and prospective controlled open studies dealing with the first-line eradication of *H. pylori*, provided by European authors were included. Accepted abstracts had to include at least 2 treatment arms, to specify the diagnosis of patients, the exact drug doses, the duration of treatment and method of eradication control (histology, culture, rapid urease test or ^{13}C -urea test). Exclusion criteria: studies coming from other continents or multi-center studies with participating centers outside Europe were not considered. Duplicate abstracts were identified and considered only once. Meta-analyses, audits and papers presenting prescribing practices were not included. Trials not specifying the type of PPI/antibiotic used, duration of treatment or incomplete data were excluded. Only eradication trials in peptic ulcer, chronic gastritis and functional dyspepsia were included, conditions in which treatment of the infection are recommended by the 1st and 2nd Maastricht consensus conference^[7,8]. Trials performed in the extragastric manifestations of the infection, MALT lymphoma and pediatric population were not part of the study. Studies using PPIs, antibiotics given intravenously or probiotics were also omitted. A flow-chart of the study is presented in Figure 1. The quality of the abstracts was verified by a validated score system including 19 items, in order to compare to the most relevant methodological details^[6]. Abstracts having a quality score under 0.50 were not accepted.

The treatment arms of the abstracts were classified and groups of similar regimens were constructed as follows: (1) PPI-based one week triple combinations; (2) Ranitidine bismuth citrate (RBC)-based one week triple combinations; (3) H₂-receptor antagonist-based triple regimens; (4) Double combinations (PPI/RBC + 1 antimicrobial); (5) Quadruple therapies (PPI + bismuth compound + 2 antimicrobials); (6) Antimicrobial-based analysis: - PPI + clarithromycin + amoxicillin or a nitroimidazole, - PPI + amoxicillin + nitroimidazole, - RBC + clarithromycin + amoxicillin or a nitroimidazole.

The results obtained were compared with the available meta-analyses based on the data of RTCs in order to assess whether the results of abstracts overlap with the latest data of the evidence-based medicine.

Statistical analysis

The eradication regimens were pooled into groups shown above. The data were entered in an Excel database which

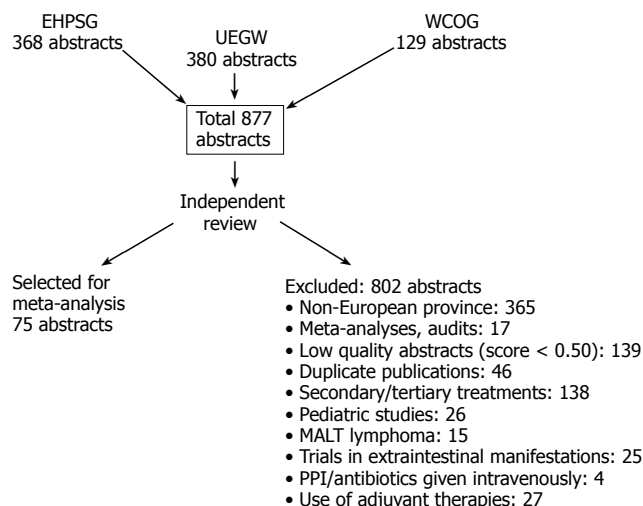


Figure 1 Flow-chart of the meta-analysis.

was checked by hand for errors. While the 'per protocol' (PP) data were given only in a part of the abstracts, the results on 'intention-to-treat' (ITT) basis were taken into consideration. The groups were checked for homogeneity by the chi-square test^[9]. The 'pooled eradication rate' (PER) of the groups including all treatment arms of similar regimens (i.e. groups 1-6) was calculated using equal effect analysis, assuming that different populations have the same outcome (N-weighted calculation). Differences between the groups were assessed by the chi-square test and the level of $P = 0.05$ was considered significant. The 95% confidence intervals (CI) were calculated. Within the groups 1-6, from the comparative trials the individual Peto odds ratios (OR) were estimated and the combined ORs were assessed assuming a random effect model^[9]. The change over time of the eradication rates of PPI-based therapies was performed by calculating the PERs for each year of the period studied (1997-2004). The statistical formulae were used as described in the literature^[9,10]. The statistical work was performed using the 'Statistica 9.0' software (Tulsa, Oklahoma, USA).

RESULTS

General information

In the post-Maastricht period, between 1997-2004, 877 papers dealing with the therapy of *H. pylori* infection were presented during the selected meetings, from which 75 met the inclusion criteria. The accepted abstracts included 188 study arms and 15 634 patients. Eight hundred and one abstracts were excluded for the reasons shown in the flow-chart. The accepted abstracts had a mean quality score of 0.66 (95% CI: 0.52-0.77). Data on the homogeneity of the groups are given in the text and Tables 1-5.

Results of primary treatment

PPI-based one week triple regimens: Most of the authors adhered to the provisions of the Maastricht 1 consensus and administered PPI-based triple regimens as first-line therapy^[12-15,17-19,21-30,32-65,78-85]. The PERs of the PPI-

Table 1 Effect of PPI-based 1 wk triple therapies on the eradication of *H pylori* in European countries (pooled estimation of all treatment arms)

PPI + 2AB	Cases (n)	Studies (n)	Treatment arms (n)	PER (%)	95% CI	P ¹	References
Omeprazole	7234	42	78	78.9	77.6-81.1		13-15, 17-19, 21-24, 26, 29, 37, 41, 43, 44, 49, 53, 57-63, 66-68, 73-76, 78-85
Lansoprazole	1048	12	17	82.9	79.2-81.1	0.95	12, 18, 22, 24, 25, 28, 32-38, 65
Pantoprazole	1216	9	13	80.1	76.5-83.6	0.84	27, 39-42, 63-65, 77
Rabeprazole	884	8	15	83.9	79.1-88.8	0.88	39, 49-56
Esomeprazole	752	6	14	81.1	80.3-88.0	0.80	43-48
All PPI	11134	77	136	81.4	78.5-84.5		

¹Omeprazole *vs* other PPIs; Test of homogeneity: Omeprazole, $\chi^2 = 6.12$, $P = 0.08$; Lansoprazole, $\chi^2 = 5.56$, $P = 0.09$; Pantoprazole, $\chi^2 = 1.46$, $P = 0.22$; Rabeprazole, $\chi^2 = 3.45$, $P = 0.33$; Esomeprazole, $\chi^2 = 1.96$, $P = 0.38$.

Table 2 Results of the direct comparative trials of the PPI-based 1-wk triple combinations

PPI comparison	Cases (n)	Studies (n)	Treatment arms (n)	PER 95% CI	P	OR (95% CI)	References
Omeprazole <i>vs</i> Lansoprazole	601	5	9	87.3 (81.7-92.3)	0.22	0.83 (0.6-1.1)	32-37, 39
Omeprazole <i>vs</i> Pantoprazole	83	1	1	89.0 (80.9-97.6)	0.24	0.61 (0.27-1.40)	39
Omeprazole <i>vs</i> Esomeprazole	253	2	2	90.5 (84.6-96.4)	0.71	0.91 (0.53-1.55)	43, 44
Omeprazole <i>vs</i> Rabeprazole	163	1	2	82.5 (74.2-90.8)	0.61	1.24 (1.00-1.48)	53
Lansoprazole <i>vs</i> Pantoprazole	567	5	9	83.9 (75.0-92.3)			
Pantoprazole <i>vs</i> Esomeprazole	85	1	1	79.6 (71.5-87.6)			
Esomeprazole <i>vs</i> Rabeprazole	256	2	2	91.5 (86.3-96.7)			
Rabeprazole <i>vs</i> Esomeprazole	163	1	2	88.5 (81.3-95.7)			

Test of homogeneity: Omeprazole/Lansoprazole, $\chi^2 = 2.76$, $P = 0.1$; Omeprazole/Pantoprazole, $\chi^2 = 2.69$, $P = 0.12$; Omeprazole/Rabeprazole, $\chi^2 = 2.45$, $P = 0.12$; Omeprazole/Esomeprazole, $\chi^2 = 0.12$, $P = 0.72$.

Table 3 Changes over time of the eradication rates of PPI-based one-triple therapies between 1997-2004

Year references	Cases (n)	Studies (n)	Treatment arms (n)	PER (%)	95% CI	P ¹	References
1997	3216	16	34	80.2	75.8-84.7		12,13,49,58,59,61-65,71-73,77,78,85
1998	1117	11	18	82.0	76.4-87.4	0.59	15,17,21-23,34-36,39
1999	1771	9	14	77.2	71.6-82.7	0.34	24-26,28,40,41,60,67,74
2000	1683	10	13	82.1	78.0-86.1	0.56	27,29,30,37,38,42,43,50,70
2001	1002	8	11	80.7	73.4-87.9	0.9	51-54,68,69,75,84
2002	904	4	7	87.1	80.8-93.4	0.12	41,55,57,76
2003	553	2	4	78.2	67.2-89.4	0.67	45,46
2004	468	3	5	82.6	73.5-91.6	0.65	47,48,56

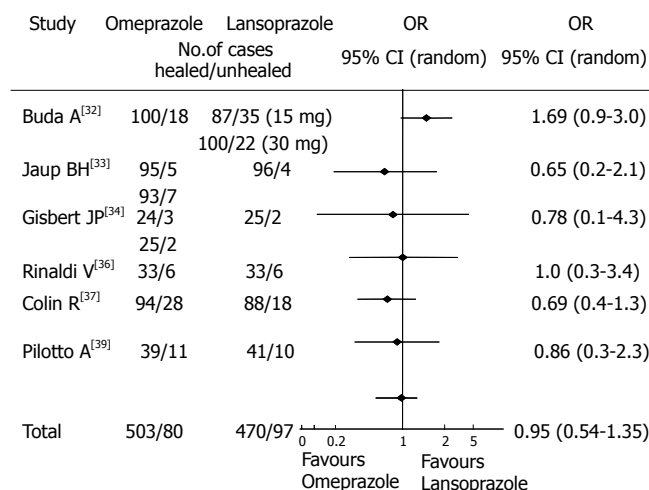
¹P: 1997 *vs* other years; $P = 0.04 < 0.05$ 2002 *vs* 1999; no other significant difference in between-year comparisons; Test of homogeneity: 1997, $\chi^2 = 9.98$, $P = 0.19$; 1998, $\chi^2 = 0.71$, $P = 0.39$; 1999, $\chi^2 = 3.0$, $P = 0.08$; 2000, $\chi^2 = 5.17$, $P = 0.15$; 2001, $\chi^2 = 0.15$, $P = 0.72$; 2002, $\chi^2 = 0.2$, $P = 0.7$; 2003, $\chi^2 = 0.14$, $P = 0.8$; 2004, $\chi^2 = 0.14$, $P = 0.8$.

based regimens and homogeneity values are given in Table 1. The overall efficacy of the PPI-based one-week triple therapies was 81.4% (95% CI: 78.5-84.5). Therapeutic experience was gained with all 5 commercially available PPI. There was no statistically significant difference of the PERs between the groups. All PPIs obtained eradication rates around 80% on ITT basis. In comparative studies, using omeprazole as the index drug, the combined ORs showed equivalence to the other PPIs (Table 2). An example is given in Figure 2 representing the results of comparative trials using omeprazole and lansoprazole-based triple regimens. The rates of *H pylori* eradication by the PPI-

based triple therapies from 1997 to 2004 are presented in Table 3. No change over time of the eradication rates was recorded.

RBC-based triple regimens: They were administered to 2051 patients in 24 studies/27 treatment arms and obtained a PER of 78.5% (95% CI: 70.5-84.3)^[11-31]. (Test for homogeneity: χ^2 : 2.79, $P = 0.16$). The OR for the effect of PPI's *vs* RBC in triple combinations was 1.10 (0.92-1.30). Including all treatment arms, there was no significant difference of the PERs between PPI- and RBC-based triple regimens ($P = 0.28$).

H₂-receptor antagonist-based triple regimens: They



$r = 5$, $\chi^2 = 1.43$, $P = 0.92$

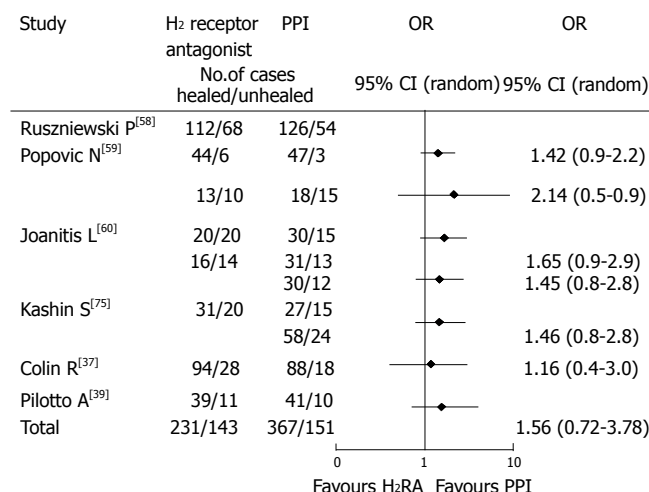
Figure 2 Omeprazole vs lansoprazole-based triple therapies: results of the comparative trials (Peto graph of the individual and combined ORs).

were given to 374 cases for 7-10-14 d in 4 studies/8 treatment arms,^[58-60,75] and obtained 64.1% (52.6-75.6) eradication, which was significantly lower as compared to PPI-based regimens (74.4%, 95% CI: 59.5-84.8) $P = 0.028 < 0.05$. (Test for homogeneity: $\chi^2: 0.21$ $P = 0.56$.) The OR for the effect of PPI *vs* H₂-RA based regimens was 1.55 (0.72-3.78) (Figure 3). However, these groups were heterogeneous (Figure 3).

Double combinations: PPI + 1 antimicrobial schedules given for 7-14 d were administered to 1051 cases in 10 studies/14 treatment arms and achieved PER of 55.0% (38.6-71.4)^[67-77]. (Test for homogeneity: $\chi^2: 2.47$, $P = 0.12$.) In comparative trials, triple combinations were efficient in 82.48% (75.4-89.3) ($P = 0.000 < 0.05$), the combined OR favoring triple combinations (4.90, 95% CI: 2.36-9.70) (Figure 4). The groups were heterogeneous (see Figure 4). RBC-based dual therapies lasting 7-14 d were given to 268 cases in 4 studies/4 treatment arms^[12,18,77,78] and obtained 77.6% (69.6-85.5) PER, which was not significantly lower than the rates of triple RBC combinations (80.8%, 72.9-88.5) ($P = 0.43$). (Test for homogeneity: $\chi^2: 0.16$ $P = 0.60$.) In direct comparative trials, the OR for the effect of RBC double *vs* triple combinations was 0.83 (0.51-1.33).

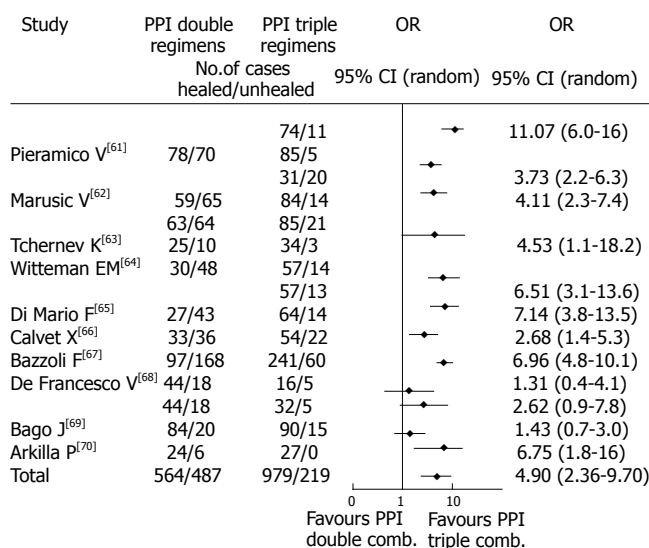
Quadruple therapies: They were administered as first-line regimens to 611 cases in 10 treatment arms, and the PER was 82.6% (76.0-89.2)^[76,79-82]. (Test for homogeneity: $\chi^2: 0.13$, $P = 0.61$.) In comparative trials, triple therapy's PER was 81.2% (76.4-86.0) ($P = 0.07$). The combined OR for the effect of triple *vs* quadruple therapies was 0.80 (0.62-1.03) (80-82).

Antibiotic combinations: The eradication rates obtained with PPI + clarithromycin or either amoxicillin or nitroimidazole regimens compared with PPI + nitroimidazole + amoxicillin are presented in Table 4 (all treatment arms). Clarithromycin + amoxicillin or a nitroimidazole containing regimens were significantly more efficient than amoxicillin + nitroimidazole combinations ($P = 0.01 < 0.05$ and $P = 0.001 < 0.05$, respectively) (Table 4) and the OR favored the former therapies over the latter (Table 5). There



$r = 5$, $\chi^2 = 16.73$, $P = 0.005 < 0.05$

Figure 3 H₂-receptor antagonists and PPI-based triple regimens: results of the comparative trials (Peto graph of the individual and combined ORs).



$r = 11$, $\chi^2 = 126.8$, $P = 0.0000 < 0.05$

Figure 4 PPI-based double and triple regimens: results of the comparative trials (Peto graph of the individual and combined ORs).

was no significant difference in the efficiency rate of RBC + clarithromycin + amoxicillin and RBC + clarithromycin + nitroimidazole regimens ($P = 0.90$), the combined OR values indicating these combinations were equivalent (OR:1.03, 95% CI: 0.65-1.38).

DISCUSSION

In the present study, we assessed the eradication rates of different anti-*H. pylori*-regimens given in European countries in the post-Maastricht period. Several meta-analyses evaluated the efficacy of the eradication regimens^[1-3,87-91,93,95,96]. To our knowledge, this is the first meta-analysis based solely on abstracts coming from the European countries, thus exploring a previously unexplored database. Our purpose was to assess whether or not these

Table 4 The effect of antibiotic combinations on the eradication of *H pylori* in European countries (pooled estimation of all treatment arms)

Antibiotic combination	Cases (n)	Studies (n)	Treatment arms (n)	PER %	95% CI	P	References
PPI + C + A	5610	40	49	81.3	77.8-84.8	0.96 ¹	12,14,22,24,26,31-34,36,39-42, 44-46,48,52,54-56,58,60-70,72-79,79
PPI + C + N	2080	25	27	83.8	81.7-85.9		14,23,25,27,28,32-34,39,43,44,47,48, 57,62,67-69,71-73,76,78
PPI + A + N	1577	15	19	73.5	66.6-78.5	0.01 ² < 0.05 0.001 ³ < 0.05	11,16,33,38,39,41,54-56,68,69,73, 78,79
RBC + C + A	589	8	9	82.6	76.2-89.0	0.74 ⁴	12-14,17,22-26,30
RBC + C + N	1285	15	17	78.0	71.4-84.6		10,11,13-15,17-19,21,23-25,27,28,30
RBC + A + N	186	1	1	76.5	67.3-88.7		16,17

Test of homogeneity: PPI + C + A, $\chi^2 = 1.13$, $P = 0.28$; PPI + C + N, $\chi^2 = 4.47$, $P = 0.03 < 0.05$; PPI + A + N, $\chi^2 = 1.07$, $P = 0.29$; RBC + C + A, $\chi^2 = 2.58$, $P = 0.11$; RBC + C + N, $\chi^2 = 1.49$, $P = 0.47$; RBC + A + N, $\chi^2 = 3.12$, $P = 0.1$. C: clarithromycin; A: amoxicillin; N: nitroimidazole, PPI: proton pump inhibitors; RBC: ranitidine bismuth citrate. Notes: ¹ PPI + C + A *vs* PPI + C + N; ² PPI + C + A *vs* PPI + A + N; ³ PPI + C + N *vs* PPI + A + N; ⁴ RBC + C + A *vs* RBC + C + N; ⁵ RBC + C + CA *vs* RBC + A + N.

Table 5 Results of the direct comparative trials of different antibiotic combinations on the eradication of *H pylori* in European countries

Antibiotic combination	Cases (n)	Studies (n)	Treatment arms (n)	PER %	95% CI	P ¹	OR	References
PPI + C + A <i>vs</i> PPI + C + N	2155	18	22	80.8	76.8-84.8	0.61	1.0 (0.90-1.20)	14,17,21,23,32-35,41,48, 68, 69,71-74,78
PPI + C + A <i>vs</i> PPI + A + N	1172	10	12	81.5	77.7-85.3	0.000 < 0.05	1.78 (1.46-2.16)	26,29,44,54-56,68,69, 73,79
PPI + C + N <i>vs</i> PPI + A + N	476	5	5	70.9	56.3-85.4	0.96	0.99 (0.77-1.29)	41,54,68,69,74
RBC + C + A <i>vs</i> RBC + C + N	122	2	3	82.4	73.6-91.1	0.90	1.03 (0.61-1.75)	13,30
RBC + C + N	166	2	2	80.9	72.4-89.4			

Test of homogeneity: PPI + C + A *vs* PPI + C + N, $\chi^2 = 0.01$, $P = 0.92$; PPI + C + A *vs* PPI + A + N, $\chi^2 = 3.26$, $P = 0.07$; PPI + C + N/PPI + A + N, $\chi^2 = 0.01$, $P = 0.99$; RBC + C + A/RBC + C + N, $\chi^2 = 2.58$, $P = 0.11$. PPI: proton pump inhibitors, A: amoxicillin; C: clarithromycin; N: nitroimidazoles; RBC: ranitidine bismuth citrate.

results are comparable with the results of the latest meta-analyses. According to our data most of the European authors followed the recommendations of the Maastricht 1-2 consensus meetings^[7,8]. Thus, the PPI-based triple therapies were the most frequently used regimens. The PER of all treatment branches using these combinations was comparable with the earlier meta-analyses. In the first such review evaluating the pre-Maastricht era studies (1992-1996), a PER of 78.3% (64.6%-88.4%) was obtained^[86]. In the largest meta-analyses published thus far, 666 publications summarizing 53 228 patients were reviewed and the PER of different PPI-based combinations was between 80%-85%, which is also compatible with our data^[2]. In another study^[87], in 22 RCTs performed before 1999, the PER of PPI + clarithromycin/amoxicillin or metronidazole regimens was 81% (95% CI: 79-83). No differences of the PERs of the available PPIs were observed in the eradication of *H pylori* and in direct comparative trials, the OR analysis showed that neither of the PPIs was favored. This is compatible with the recent data showing that in comparative trials, the efficacy of omeprazole and pantoprazole^[88,89], rabeprazole^[90] lansoprazole^[88] and esomeprazole^[91] was similar. There are data suggesting a decrease in

the eradication rate of PPI-based triple regimens in recent years^[92]; however, as shown in Table 3, such changes over time could not be confirmed in our analysis. The PER of RBC-based triple regimens is similar with that of PPIs and in comparative trials, the OR values indicated these therapies were equivalent, which is in accordance with a meta-analysis^[93], and is in agreement with the Maastricht consensus recommendations.

According to our data, H₂-receptor antagonist-based therapies were inferior to either PPI or RBC-containing regimens and the ORs favored the later schedules. The heterogeneity of the groups, however, needs caution in the interpretation of these results. In a meta-analysis, the overall efficacy of H₂ receptor antagonists and PPIs as adjuvants for *H pylori* therapy was similar (78% *vs* 81% on ITT basis); including clarithromycin in the regimens, a non-significant trend favoring H₂ receptor antagonists was observed (OR1.14, 95% CI: 0.76-1.71)^[95]. In another study, the overall efficacy of PPI was superior to H₂ receptor antagonists (74% *vs* 69%, OR: 1.31, 95% CI: 1.09-1.58); however, prescribing high doses of H₂ blockers could improve the results^[96]. This discrepancy warrants further study while in case of equivalence, the use of H₂-receptor

blockers would have pharmaco-economic advantages.

PPI-based double combinations^[61-71] were clearly inferior to triple regimens, which is in accordance with the evidence-based data^[2] and they are probably not more recommended in the first-line treatment of the infection. Interestingly, RBC-based double combinations have similar efficacy with the triple regimens^[11,16,71,72]. However, no meta-analysis addressed this issue. Here again, heterogeneity of the groups might influence the results. Quadruple therapies have been demonstrated to be very effective and safe either as first or second-line therapies. Concerns have been raised regarding the optimal duration of treatment, efficiency and side effects, because prolonging the therapy is not cost-effective, the gain in effectiveness is modest and the tolerability could be impaired. Overall, quadruple regimens were not better than triple therapies^[70,73-76] and this is in agreement with a recent meta-analysis, where these therapies seem to be roughly equivalent (81% vs 78%, OR: 0.81, 95% CI: 0.55-1.20)^[97]. The Maastricht 2 Consensus recommended their use as a second-line treatment but, and according to a recent meta-analysis, they could equally be used as first-line, based on their efficacy^[5].

The choice of antimicrobials is of crucial importance while resistance to antibiotics is a major determinant of the eradication outcome^[3,97]. In our study, clarithromycin + amoxicillin or a nitroimidazole combination have a superior yield as compared to amoxicillin + a nitroimidazole combination, which is in accordance with a meta-analysis^[87].

We conclude, that selection of high-quality abstracts of major gastroenterologic meetings constitutes a valuable pool of data for meta-analysis. The results obtained by us are mainly in accordance with the existing data. Some discrepancies must be resolved by further studies. Primary treatment for *H pylori* infection is usually successful in over 80% of the patients; however, there is no ideal first-line regimen^[98,99]. Nevertheless, it was not our intention to analyze all aspects of the eradication therapy, which would be far beyond the scope and extent of this work. Other details, i.e. data on the influence of initial diagnosis (i.e. gastric or duodenal ulcer, functional dyspepsia), treatment duration, antimicrobial resistance, newer antibiotic combinations, rescue treatments, geographical distribution of the eradication results merit further analysis and updated research.

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BASIC RESEARCH

Taurolithocholate impairs bile canalicular motility and canalicular bile secretion in isolated rat hepatocyte couplets

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Abstract

AIM: To investigate the effects of taurolithocholate (TLC) on the canalicular motility in isolated rat hepatocyte couplets (IRHC).

METHODS: TLC was added to IRHC at concentrations of 10 and 50 $\mu\text{mol/L}$, respectively. In each group, five time-lapse movies containing 3 representative bile canaliculi were taken under phase-contrast microscopy for 12 h. The number of bile canalicular contractions and the intervals between consecutive canalicular contractions were calculated. Furthermore, the effects of TLC on IRHC were examined by transmission electron microscopy.

RESULTS: The bile canalicular contractions were spontaneous and forceful in the controls. Active vesicular movement was observed in the pericanalicular region. Immediately after the addition of TLC, the bile canaliculi were deformed, and canalicular bile was incorporated into the vacuoles. The canaliculi were gradually dilated, and canalicular contractions were markedly inhibited by TLC. The vesicular movements became extremely slow in the pericanalicular region. The number of canalicular contractions significantly decreased in the TLC-treated groups, as compared with that in the controls. The time intervals were prolonged, as the TLC dosage increased, indicating that bile secretion into the canaliculi was impaired with TLC. Transmission electron microscopy revealed the lamellar transformation of the canalicular membranes in IRHC treated with TLC.

CONCLUSION: TLC impairs both the bile canalicular contractions and the canalicular bile secretion, possibly by acting directly on the canalicular membranes in TLC-induced cholestasis.

INTRODUCTION

The concept that pericanalicular actin filaments may subserve a contractile function to facilitate bile flow was first proposed in 1974^[1]. Contractile proteins are found to be present throughout the cytoplasm in hepatocytes and are particularly numerous in the pericanalicular region by electron microscopy^[2,3], immunohistochemistry^[4,5], and biochemical analysis^[6]. Strong evidence for bile canalicular motility came from *in vitro* studies using isolated rat hepatocyte couplets (IRHC)^[7,8], which indicated dynamic contractions of the bile canaliculi. The canalicular motility is impaired by actin inhibitors such as cytochalasin B and phalloidin^[9,10]. Taurocholate, a choleretic bile acid, also increases the contraction rate, thus providing further support for a role of canalicular contractions in the bile secretory mechanism^[11]. More importantly, canalicular contractions have been demonstrated in living rats^[12], and this finding substantiates a number of *in vitro* data regarding the mechanisms of bile canalicular contractions.

Lithocholic acid, a hydrophobic secondary bile acid, is well known to cause intrahepatic cholestasis^[13]. There have been extensive studies on the mechanisms of lithocholate-induced cholestasis in animals. Lithocholate diminishes both the bile acid-dependent and independent bile flow^[14]. Regarding the mechanisms of the bile acid-independent bile flow, it has been explained by the inhibition of Na^+ , K^+ -ATPase^[15,16], the inhibition of gap junction permeability^[17], and the impairment of hepatic transporters such as the bile acid export pump (Bsep)^[18-20]. In humans, elevated levels of lithocholic acid are found in patients with chronic cholestatic liver disease^[21,22]. From an ultrastructural point

of view, the lamellar transformation of the canalicular membranes reported by Miyai *et al.* has been considered the hallmark of lithocholic acid-induced cholestasis^[23,24]. To elucidate the precise mechanism of cholestasis, we investigated the direct effects of tauroolithocholate (TLC) on the canalicular motilities in IRHC using time-lapse cine-photomicrography and transmission electron micrography.

MATERIALS AND METHODS

Liver cell culture

Wistar strain female rats, weighing 170-230 g, were used. These rats were fed with a laboratory pellet rat diet and tap water *ad libitum*. All animals were given humane care in compliance with institutional guidelines. Primary isolated hepatocytes were prepared using the same method reported previously^[8,9]. Briefly, liver perfusion and dissociation were based on the method of Seglen^[25] as modified by Laishes and Williams^[26]. After an initial washout perfusion was performed through the portal vein using Ca^{2+} and Mg^{2+} free Hanks balanced salt solution containing 0.5 mmol/L EGTA, the rat livers were then perfused with L-15 medium containing 0.05% type I collagenase (Sigma Chemical Company). One million isolated hepatocytes were inoculated into a 60 mm Corning culture dish (Corning Glass Works, Corning, NY), and then maintained with L-15 medium which contained 10% fetal bovine serum, 10 mmol/L HEPES, penicillin (100 U/L) and streptomycin (100 U/L). The viability of isolated hepatocytes by trypan blue exclusion test was approximately 95%. These cells were preincubated at 38°C for 4 h to facilitate cell attachment to the bottom of the dish and to allow time for recovery from the cell isolation procedure. The incompletely separated groups of cells, especially couplets and triplets, were selected for this experiment.

Time-lapse cinephotomicrography

A Nikon inverted microscope (Diaphot-TMD) with phase-contrast optics and a 16 mm movie camera (H16, RX-5 Bolex) operated by a Nikon cine auto-timer with a drive system (CFMA) were used. This equipment was housed in a temperature-controlled room maintained at 38°C. Time-lapse movies were taken at a speed of 1 frame/15 s for 12 h after the addition of TLC. Five time-lapse movies containing 3 representative bile canaliculi from 5 rats were taken for each of the experimental and control groups.

Experimental design and methods of analysis

In the experimental groups, sodium tauroolithocholate (grade A, purity > 99%, Calbiochem, San Diego, CA) was added to the culture media at concentrations of 10 and 50 $\mu\text{mol/L}$, respectively. The TLC solution was prepared from the stock solution containing 20 mmol/L TLC dissolved in propylene glycol. In the controls, the same solution without TLC was added to the culture media. Three representative bile canaliculi in each movie were used for analysis. A total of 2880 frames, i.e., 12 h of real time were examined in each canaliculus using an analytic movie projector (Photo-Optical Data Analyzed 2240A,

MK-V, L-W International, Woodland Hills, CA). Bile canalicular contraction was defined as a visible decrease in canalicular diameter. The minimum size of the canalicular lumen was taken as the end point of the contraction. Analysis of the movies was performed using the frame numbers of the movie films at the point of beginning and end of the contractions. The time intervals between adjacent contractions were calculated as the difference in the frame counts between consecutive contractions. These frame counts were then converted into real time.

Statistical analysis

The frame numbers obtained from all the movies were stored and analyzed by a personal computer. The number of bile canalicular contractions was determined for 3 canaliculi from each movie of 5 rats and evaluated by analysis of variance test for each dose of TLC used. The intervals between consecutive canalicular contractions were calculated and compiled to make a histogram. The histograms of 10 and 50 $\mu\text{mol/L}$ TLC and controls were assessed statistically, presuming that these data have a Poisson distribution as reported previously^[8,11].

Electron microscopy

Representative samples of IRHC after the addition of 10 and 50 $\mu\text{mol/L}$ TLC and the controls were examined by transmission electron microscopy. The cells cultured on plastic cover slips were fixed in universal fixative^[27] at 1, 3 and 6 h after the addition of either TLC or propylene glycol. For postfixation, 1% osmium tetroxide in 0.1 mol/L cacodylate buffer (pH 7.4) for 30 min was used. The cells were dehydrated in a graded series of alcohol and embedded in Epon 812. Ultrathin sections were cut using LKB ultramicrotome and stained with Sato's lead solution^[28]. The specimens were examined using a Philips EM 400T electron microscope (Philips Electronic Instruments Inc., Mahwah, N.J.).

RESULTS

Remarkable motilities of isolated hepatocyte couplets and triplets were observed in the time-lapse movies. In the controls, bile canaliculi revealed spontaneous and forceful contractions (Figure 1). The canalicular contractions were most striking for cell motility, while Brownian-like movement and cell ruffling were also seen. Active movements of small particles, which may represent cytoplasmic vesicles and vacuoles, were noted in the pericanalicular regions of the cytoplasm. TLC-treated hepatocytes manifested a marked reaction to this bile acid as compared with the controls. Immediately after the addition of TLC, the bile canaliculi were deformed, and canalicular bile was incorporated into the vacuoles in hepatocyte cytoplasm. The canaliculi were gradually dilated, and canalicular contractions were markedly inhibited by TLC (Figure 2). This phenomenon was most prominent with a high dose (50 $\mu\text{mol/L}$) of TLC. Some bile canaliculi showed only a few contractions approximately 6 h after the addition of TLC. The pericanalicular movements of the small particles observed by phase-contrast microscopy

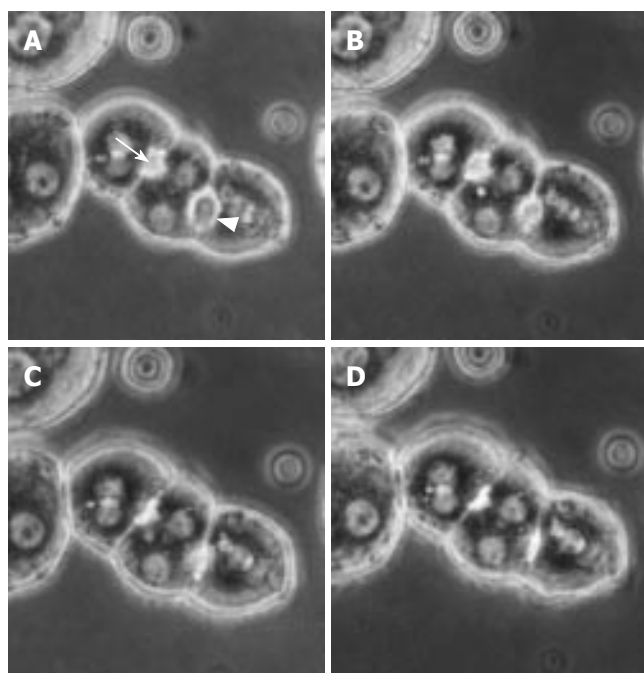


Figure 1 Representative phase contrast micrographs of an isolated hepatocyte triplet in the controls. Figures A, B, C and D show a sequence of the bile canalicular contractions. A bile canaliculus indicated by an arrowhead started to contract (A) and completed contraction (D). Another canaliculus indicated by an arrow started its contraction (B) and completed contraction (C). These canaliculus contractions were forceful and repetitive.

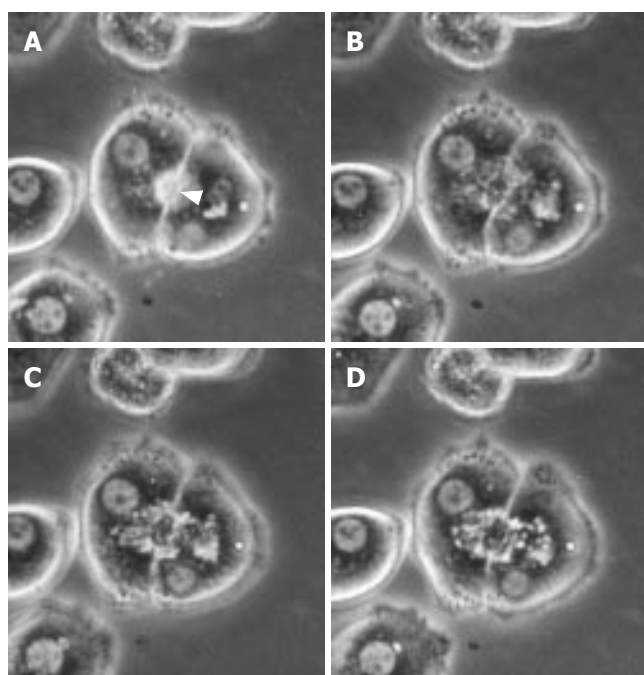


Figure 2 Representative phase contrast micrographs of an isolated hepatocyte couplet in the taurolicholate (TLC)-treated groups (50 $\mu\text{mol/L}$). Prior to addition (A) and after the addition of TLC (B, C, D). A canaliculus (arrowhead) between two hepatocytes (A) was deformed, and canalicular bile was incorporated into the vacuoles in the hepatocyte cytoplasm immediately after the addition of TLC (B). Thereafter, the bile canaliculi were gradually dilated, and the canaliculus contractions were markedly impaired (C, D). In the TLC-treated groups, the movements of small particles became extremely slow, and cytoplasmic vesicles and vacuoles were noted in the pericanalicular region.

became extremely slow with the progression of time. A number of cytoplasmic vesicles and vacuoles were found

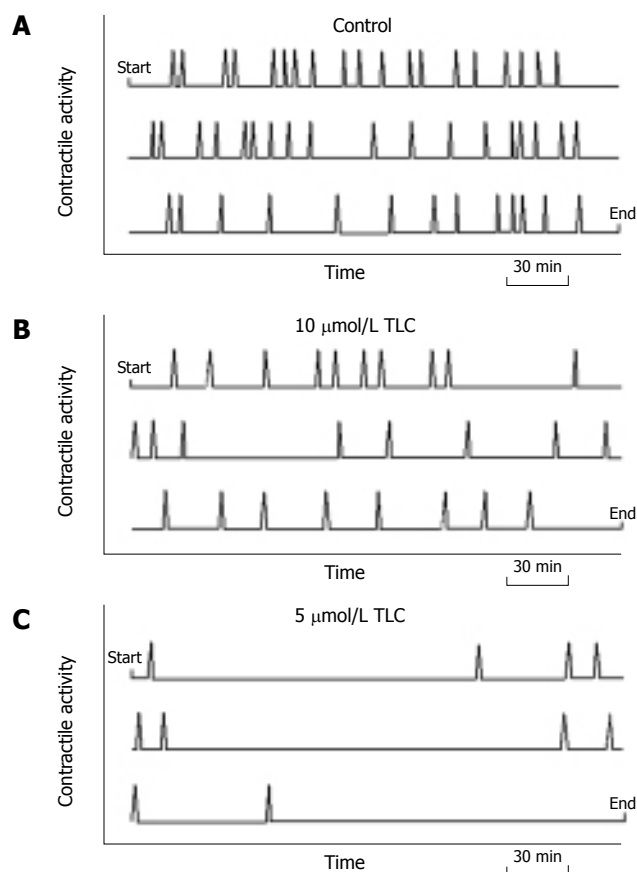


Figure 3 Graphic display of the representative contraction activity of bile canaliculi. Diagrams represent the 12 h period of observation, beginning at the upper left corner and ending at the lower right corner. The canaliculus contractions (spikes) occurred at regular intervals in the control groups (A). The contraction activity visibly diminished as the TLC dosage increased (B, C).

in the pericanalicular region. The graphic displays of representative canaliculus contractions in the controls and the TLC-treated groups are shown in Figure 3. Whereas canaliculus contractions occurred at regular intervals in the control groups, the contraction activity was visibly diminished, as the TLC dosage increased.

To examine the detailed functions of the canaliculus contractions, we analyzed the number of contractions and the contraction interval for 12 h. The total contraction number of 3 bile canaliculi in the TLC-treated groups significantly decreased to 82.4 ± 6.1 (mean \pm SE) at 10 $\mu\text{mol/L}$ and 30.2 ± 3.1 at 50 $\mu\text{mol/L}$, as compared with that in the control groups (139.4 ± 4.5) (Figure 4). The histogram of the canaliculus contraction interval revealed the Poisson distribution. In the TLC-treated groups, there was a tendency for the peak height to decrease and shift to the right. In other words, the time intervals between adjacent contractions were prolonged, as the TLC dosage increased. The calculated theoretical peak values, which reflect the maximum likelihood of the next contraction, were 4.7 min in the control groups, 5.7 min at 10 $\mu\text{mol/L}$ and 7.0 min at 50 $\mu\text{mol/L}$ TLC (Figure 5).

Transmission electron microscopy revealed that IRHC prior to the addition of TLC showed either normal or slightly dilated bile canaliculi. The canaliculi between two hepatocytes were filled with microvilli and sealed by tight junctions. A rich network of microfilaments, i.e.,

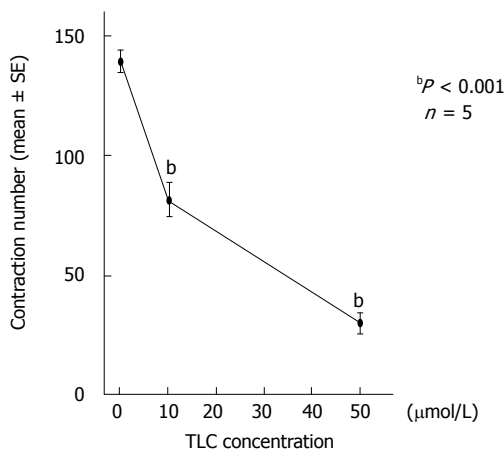


Figure 4 Effects of tauroolithocholate (TLC) on the contraction number of bile canaliculi. The total contraction number of 3 bile canaliculi for 12 h was 139.4 ± 4.5 (mean \pm SE) in the controls, and it significantly decreased to 82.4 ± 6.1 at 10 $\mu\text{mol/L}$ TLC and 30.2 ± 3.1 at 50 $\mu\text{mol/L}$. The contraction number markedly decreased as the TLC dosage increased.

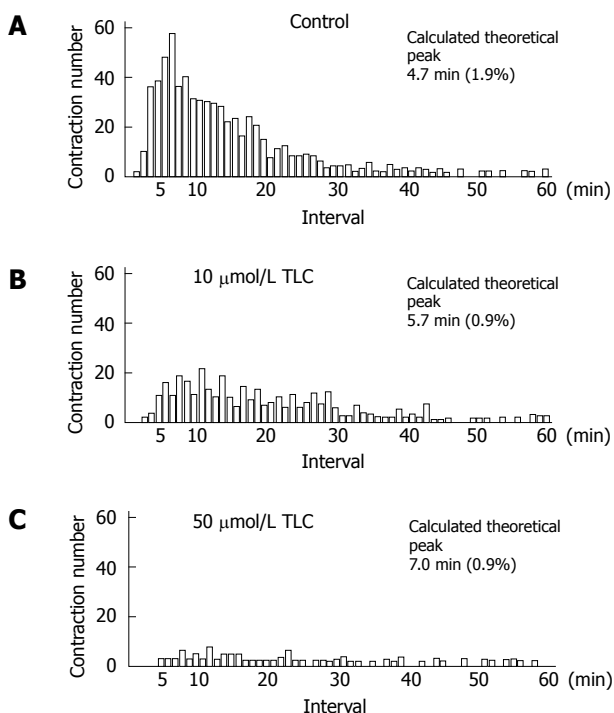


Figure 5 Effects of tauroolithocholate (TLC) on the contraction interval of bile canaliculi. The histogram of the canalicular contraction interval revealed Poisson distribution. In the TLC-treated groups, there was a tendency for the peak height to decrease and shift to the right. The calculated theoretical peak values for each dose were 4.7 min in the control groups (A), 5.7 min at 10 $\mu\text{mol/L}$ TLC (B) and 7.0 min at 50 $\mu\text{mol/L}$ (C). Normalized errors are indicated in parentheses.

actin filaments were recognized around the canaliculi. It was noted that TLC caused characteristic lamellar transformation of the bile canalicular membranes (Figure 6). This lamellar structure became more prominent and widely spread with the progression of time after the addition of TLC. The bile canaliculi were deformed and tended to become dilated with the loss of microvilli. Electron dense membranous structures of the biliary materials were found to be incorporated into the

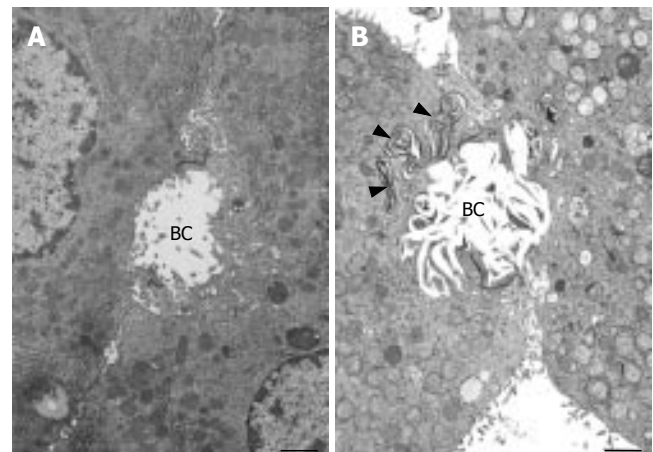


Figure 6 Transmission electron micrographs of isolated hepatocytes. A bar indicates 1 μm . A: Normal hepatocytes prior to the addition of tauroolithocholate (TLC). A bile canaliculus (BC) was slightly dilated and filled with microvilli. B: 12 h after the addition of 50 $\mu\text{mol/L}$ TLC. Note the characteristic lamellar transformation of the bile canalicular membranes. A bile canaliculus (BC) was deformed and dilated with a loss of microvilli. Electron dense membranous structures of biliary materials (arrowheads) were found in the vacuoles in the pericanalicular region.

vacuoles in the hepatocyte cytoplasm, especially in the pericanalicular region.

DISCUSSION

The present study using time-lapse movies permits the continuous recording of the dynamic cell motilities in IRHC. The bile canaliculi between adjacent isolated hepatocytes manifested a marked reaction to TLC. The canaliculi were immediately deformed after the addition of TLC, and thereafter they became dilated with the marked impairment of the canalicular contractions. The contraction number of the canaliculi significantly decreased to 82.4 ± 6.1 at 10 $\mu\text{mol/L}$ and 30.2 ± 3.1 at 50 $\mu\text{mol/L}$, as the TLC dosage increased. Since Phillips and his co-workers demonstrated dynamic contractions of the bile canaliculi^[7,8], a number of studies using IRHC have been performed to elucidate the mechanisms of the bile canalicular contraction. The results indicate the active contraction process of the bile canaliculi mediated by the pericanalicular contractile proteins. It is notable that the contractions are Ca^{2+} , calmodulins and ATP-dependent^[4,9,10,29]. Hence, the mechanism of canalicular contraction has features in common with the actin-myosin based cytoplasmic motility behavior found in other non-muscle cells. The canalicular contractions are spontaneous but highly ordered. In triplets, when one bile canaliculus contracts, a neighboring canaliculus is likely to contract after an interval of 1.25 min, thus indicating a coordination in the contractile activity of the BC^[30]. In the present study, the BC in the control couplets and triplets showed spontaneous and forceful contractions. The total contraction number of 3 bile canaliculi was 139.4 ± 4.5 in the control groups, which is almost equal to the findings of previous reports^[9-11].

The permeability of cell membranes is greatly influenced by cholesterol, including specific changes in the membrane cholesterol affected by both the functional properties of the lipid bilayer and the function of mem-

brane-bound proteins^[31-33]. In physiological conditions, cholesterol has been viewed as a modulator of membrane fluidity and permeability^[34,35]. The alterations in membrane permeability or fluidity in the liver have been considered to be one of the mechanisms of intrahepatic cholestasis^[36]. In this regard, lithocholate-induced cholestasis is of special interest, since the canalicular membrane itself is the site of cholesterol deposition^[16,23]. A biochemical analysis of canalicular enriched isolated plasma membrane preparations revealed an eight-fold increase in cholesterol in TLC-induced intrahepatic cholestasis^[24]. It is therefore anticipated that pathological alterations in cholesterol of the canalicular membranes may affect the membrane structure and function, especially bile canalicular contractions.

In this study, a canaliculus between adjacent isolated hepatocytes manifested a marked reaction to TLC. Immediately after the addition of TLC, the bile canaliculi were deformed and gradually dilated, leading to a remarkable reduction in the canalicular contraction number. The prompt responses of the bile canaliculi to TLC suggest that TLC may directly act on the canalicular membranes, resulting in the alteration and dysfunction of the canaliculi. TLC is mainly taken up by the hepatocytes through the Na⁺ taurocholate cotransporting polypeptide (Ntcp)^[37], and the canalicular secretion of TLC is mainly mediated by Bsep^[38]. It is therefore speculated that TLC taken up by IRHC may directly act on the canalicular membranes, resulting in the formation of the lamellar structure as recognized by electron microscopy. In normal IRHC, a rich network of microfilaments is found in the pericanalicular region, and the circumferential band of microfilaments surrounding the bile canaliculi is considered to fulfill a contractile function^[4]. Since the ultrastructural alterations of the pericanalicular microfilaments were not evident after the TLC treatment in this study, it is considered that the impairment of the canalicular contraction in IRHC may thus be due to the abnormalities of the canalicular membranes themselves, without affecting the cytoskeletal organization around the bile canaliculi.

It is likely that the canalicular motility events are related to bile secretion. We analyzed the contraction interval of the bile canaliculi to elucidate the effect of TLC on the canalicular secretory function by the same method as previously reported^[11]. The histogram of the canalicular contraction interval indicates that there was a tendency for the peak height to decrease and shift to the right, as the TLC dosage increased. The calculated theoretical peak values, which reflect the maximum likelihood of the next contraction, were 4.7 min in the control groups, 5.7 min at 10 $\mu\text{mol/L}$ and 7.0 min at 50 $\mu\text{mol/L}$ TLC, indicating that the time intervals between adjacent contractions are prolonged by TLC. The bile canaliculi are the smallest biliary passages across which bile secretion occurs. Taurocholic acid causes a linear increase in bile secretion in IRHC, suggesting that canalicular contractions may be a function of canalicular bile secretion^[11]. The present study demonstrates that TLC impairs not only the bile canalicular contractions, but also canalicular bile secretion. The reduction in the contractions after a lag period probably indicates that secretion is impaired, and hence the need for contractions is reduced. Since colchicines, an

inhibitor of microtubules, also inhibits both the canalicular contractions and bile secretion in IRHC^[39], these results therefore support the view that canalicular motility is closely associated with canalicular bile secretion.

Recent research on bile secretion has focused on the characterization of hepatobiliary transporters such as Ntcp and Bsep, indicating that TLC impairs the expression of Bsep^[18-20]. Bsep is a major bile acid transporter in the liver, and mutations in Bsep result in progressive intrahepatic cholestasis. Crocenzi et al. reported that TLC impairs bile salt secretion at the canalicular level and induces the internalization of Bsep into a cytosolic vesicular compartment without affecting the cytoskeletal organization in IRHC treated with TLC^[20]. Bsep expression has also been reported to be regulated by ligands of the nuclear receptor farnesoid X receptor (FXR), and TLC decreases the expression of Bsep through FRX^[19]. Taking all of these findings into consideration, the reduction of the canalicular secretion recognized in the present study may thus be associated with the decreased expression of Bsep in addition to the alterations in membrane permeability. The relationship of Bsep expression to the canalicular membrane permeability is still unknown, so the answer to this question must await further studies. In conclusion, TLC directly alters the bile canalicular membranes, thereby impairing both the canalicular motility and canalicular bile secretion.

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BASIC RESEARCH

Evaluation of IGL-1 preservation solution using an orthotopic liver transplantation model

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Abstract

AIM: To compare, in a pig liver transplantation model, the protective effect of UW with that of IGL-1, a high-sodium preservation solution containing polyethylene glycol (PEG) as an oncotic supply.

METHODS: All livers were harvested and grafted orthotopically according to standard techniques. The livers were washed out and preserved for 7 h in IGL-1 ($n = 6$) or in UW solution ($n = 7$) at 4°C. In a sham group ($n = 4$), the livers underwent a 60-min warm ischemia at 37°C. The hepatocellular injury was assessed in organ preservation solution washed out from the graft at the end of ischemic storage (before revascularization), and in serum 2 h after reperfusion and daily for up to 6 d.

RESULTS: Livers preserved in IGL-1 solution released markedly less AST than that preserved in the UW solution before and after revascularization ($P < 0.05$). Besides, the activity of creatine kinase-BB, a marker of sinusoidal lining cells injury, was higher in the UW group than in the IGL-1 group ($P < 0.05$). Histological results showed less necrotic regions in livers preserved in IGL-1 solution; however, no difference was observed for inflammation.

CONCLUSION: IGL-1 liquid effectively protects parenchymal and non-parenchymal cells against preservation-reperfusion injuries.

INTRODUCTION

Reperfusion injury after cold preservation is associated with the occurrence of primary non-function and delayed graft function, which predisposes allografts to acute and chronic rejection^[1]. Despite these obstacles, organ transplantation has become a clinical routine for several years. Nevertheless, the need of extended ischemia times and the recruitment of marginal donors indicates that this therapy must be optimized. Consequently, it has become necessary to look for better ways to improve cold storage solutions.

The golden standard liquid for the cold preservation of abdominal organs is the University of Wisconsin (UW) solution. Although largely used and incontestably efficient, its performance is continually questioned^[2,3]. It has been reported that performances of this solution are limited by its adjunction of hydroxyethyl starch (HES) that has a hyper-aggregating effect on rat and human red blood cells^[4,5], and by its high potassium concentration that could damage cells^[6,7].

Our previous works on isolated perfused rat livers and kidneys indicates that polyethylene glycol (PEG-35) could be efficiently substituted for HES in an extracellular-like UW solution^[8,9]. The clinical use of this Na-PEG-UW solution, also known as IGL-1, suggests a superiority of IGL-1 compared to standard UW for human kidneys preservation^[10]. However, to our knowledge, no studies have been reported for liver transplantation. Therefore, we aimed to evaluate the preservative effect of IGL-1 solution using an orthotopic liver transplantation (OLT) model in pigs, and to compare its effectiveness with that of the UW solution.

MATERIALS AND METHODS

Animals

Female large white pigs, weighing 25-30 kg, were used as organ donors and recipients. All animals were given humane care and handled in compliance with French regulations. Animals were fasted for 24 h prior to the intervention but had free access to water. All animals received pre-anesthesia with tiletamine (10 mg/kg), zolazepam (7.5 mg/kg) and atropine sulfate (10 µg/kg). After intubation, inhalation anesthesia was maintained by halothane (0.5%-1%), nitrous protoxide and oxygen.

Orthotopic liver transplantation

We used a standard technique for the donor operation. Portal vein (PV), infrahepatic vena cava (IHVC) and suprahepatic vena cava (SHVC) were dissected. The hepatic artery was kept in continuity with the celiac trunk, and the abdominal aorta up to the diaphragmatic pillars. Livers were harvested and washed out *ex situ* with 2 L of chilled preservation solution through PV. The harvested grafts were then cold stored at 4°C for 7 h.

In each recipient animal, a catheter was placed into the aorta to monitor blood pressure during the operation. It has been observed that complete vascular clamping during the anhepatic phase generates severe hypotension, acidosis and hyperkalemia, which may cause the death of the animal^[11]. We, therefore, placed a porto-jugular passive bypass (PJB). Just before and during the anhepatic phase, 500 mL of Hesteril solution (60 g/L of HES in 9 g/L of NaCl) was infused into the recipient to maintain a stable hemodynamic condition. The graft was positioned in the hepatic fossa, SHVC and IHVC and PV of the donor were then anastomosed, respectively, to those of the recipient with running sutures of 4/0 Prolene (for SHVC and IHVC) and 5/0 Prolene (for PV). During portal anastomosis, the graft was flushed out by Hesteril at room temperature (200 mL, IHVC was used as a vent), to purge the preservation solution and waste metabolites accumulated throughout the cold storage period. The donor aorta was implanted end-to-side to the recipient infra-renal aorta with running 7/0 Prolene, and finally the biliary continuity was re-established by using the intra-ductal stent. Reperfusion was thus started after finishing the portal anastomosis and completed after the arterial anastomosis. After OLT, no immunosuppression was given to the animals.

Experimental groups

Animals were divided into three experimental groups. Livers of the UW group ($n = 7$) were cold stored in the original Belzer liquid without dexamethasone, insulin and antibiotics. Livers of the IGL-1 group ($n = 6$) were preserved in the same solution but with inverted concentrations of Na⁺ and K⁺ and containing PEG-35 that was substituted for HES (Table 1). A sham group ($n = 4$) was designed for the purpose of evaluating the effect of warm ischemia throughout OLT. Indeed as for recipients, after complete vascular clamping, a PJB was installed for 60 min. Livers were then rinsed with Hesteril solution and finally PV and IHVC were anastomosed.

Table 1 Composition of UW and IGL-1 preservation solutions

	UW	IGL-1
HES (mmol/L)	0.25	-
PEG-35 (mmol/L)	-	0.03
Lactobionic acid (mmol/L)	100	100
Raffinose (mmol/L)	30	30
MgSO ₄ (mmol/L)	5	5
KH ₂ PO ₄ (mmol/L)	25	25
Glutathione (mmol/L)	3	3
Adenosine (mmol/L)	5	5
Allopurinol (mmol/L)	1	1
Na ⁺ (mmol/L)	30	125
K ⁺ (mmol/L)	125	30
Osmolality (mOsm/Kg)	320	320
pH	7.2-7.4	7.2-7.4

Biochemical determinations

The follow-up was of 6 duration. In order to assess the extent of parenchymal cell damage, the release of AST and ALT in serum was monitored 2 h after reperfusion and then daily for 6 d. Kinetic measurements of enzymes were determined at $A_{340\text{ nm}}$ on a Hitachi 747 analyzer. In addition, activities of total creatine kinase (CK) and CK-BB isoenzyme^[12] were monitored in the organ preservation solution washed out from the graft at the end of ischemic storage and 2 h after reperfusion. Total CK activity was determined with a commercial kinetic UV reagent (BioMérieux, Charbonnières-les-Bains, France). CK isoenzymes were separated electrophoretically on agarose gels with a Paragon kit (Beckman instruments, France). After electrophoresis, CK isoenzymes were detected under UV light, and gels were scanned with a fluorimetric densitometer (SEBIA, France).

Histological study

Liver biopsies were performed after cold storage and on the 6th d. In sham animals, the biopsies were performed only on the 6th d. Histology was achieved on 4-µm thick paraffin-embedded sections by using conventional hematoxylin-eosin staining. The analysis focused on hepatocellular necrosis, sinusoidal dilatation and inflammatory cellular infiltration. The severity of the histological lesions were scored from 0 (normal liver) to +++ (maximal damage).

Statistical analysis

Results were expressed as mean \pm SE. Data between groups were compared using the ANOVA test, followed by the student Newman-Keul test. $P < 0.05$ was considered statistically significant.

RESULTS

All the animals survived for up to 6 d. At time of sacrifice, sites of anastomosis were carefully observed, and all were patent. The groups were comparable with regard to warm and cold ischemia.

The activities of CK-BB and AST were assessed in the first sample retrieved during initial graft rinsing. The CK-

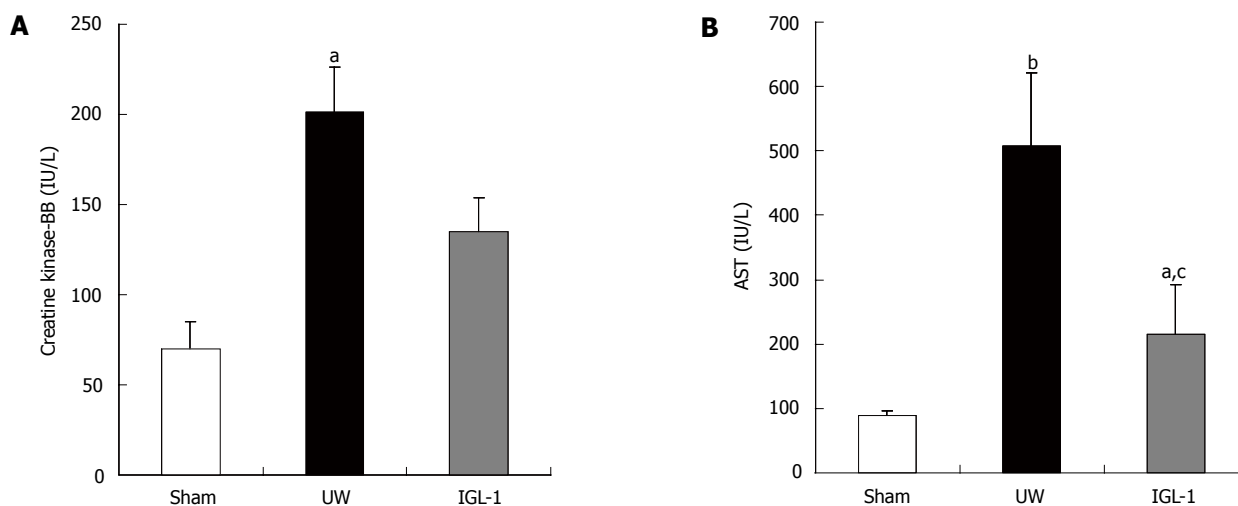


Figure 1 Creatine kinase-BB isoenzyme (A) and aspartate aminotransferase (B) activities in the preservation solution purged from the liver grafts before reperfusion. Data are expressed as mean \pm SE. ^a $P < 0.05$ vs sham; ^b $P < 0.01$ vs sham; ^c $P < 0.05$ vs UW.

Table 2 Histological results of liver specimens at the time of the animals sacrifice

Groups	Necrosis	Inflammation
UW	+++	+
	-	-
	-	+++
	-	+++
	++	++
IGL-1	+++	++
	-	+++
	-	+++
	+	+
	-	+

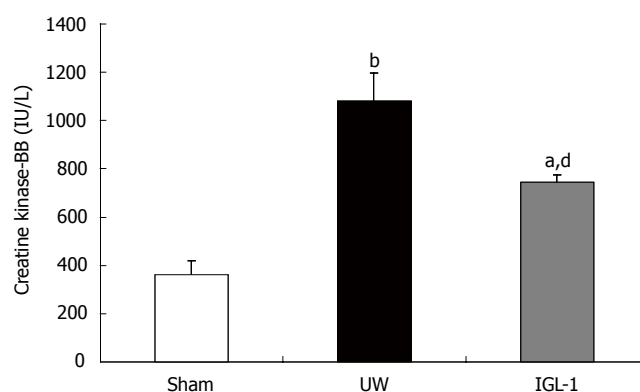


Figure 2 Serum creatine kinase-BB isoenzyme activity 2 h after reperfusion. Data are expressed as mean \pm SE. ^a $P < 0.05$ vs sham; ^b $P < 0.01$ vs sham; ^d $P < 0.01$ vs UW.

BB isoenzyme was shown to be a marker of sinusoidal lining cells injury^[12]. There was no significant difference between sham and IGL-1 groups (Figure 1A). In contrast, livers preserved in UW solution released significantly more CK-BB ($P < 0.05$) and AST (Figure 1B, $P < 0.01$) compared to the sham group. Furthermore, the IGL-1-preserved livers released obviously less AST than those preserved in UW liquid (215 ± 78 vs 507 ± 115 IU/L, $P < 0.05$).

Creatine kinase-BB isoenzyme was also monitored 2 h after reperfusion (Figure 2). The data support the view that the levels of this marker after reperfusion are related to the degree of preservation injury. Indeed, livers preserved in UW released significantly more CK-BB (1081 ± 117 IU/L) than livers of sham (361 ± 59 IU/L, $P < 0.01$) and those cold stored in IGL-1 (746 ± 29 IU/L, $P < 0.01$).

After transplantation, the highest activities of AST (Figure 3A) were reached at d 1-2 in both UW and IGL-1 groups ($P < 0.01$ vs sham). They were significantly higher in the UW vs IGL-1 group (1275 ± 215 vs 847 ± 103 IU/L at d 1 and 1046 ± 214 vs 658 ± 122 IU/L at d 2, $P < 0.05$). When livers were preserved in IGL-1 liquid, the activity of ALT (Figure 3B) did not vary at the different times of reperfusion. In contrast, ALT released by livers preserved

in the UW peaked at d 2, although there were no statistical differences between the experimental groups.

The histological study (Figure 4 and Table 2) showed no cellular alterations in the sham group. Livers preserved in IGL-1 liquid were normal with mild necrosis. In contrast, the UW group revealed multiple and extensive areas of hepatocyte necrosis.

DISCUSSION

The prevention of preservation injury is still a subject of interest, the main target of which is the reduction of primary graft non-function or sub-optimal graft function. In this study, the preservative effects of UW and IGL-1 (a PEG-high Na-UW solution) were investigated by using a pig OLT model. The results showed that liver cells integrity was best preserved with IGL-1 liquid.

Our previous studies demonstrated that the simple inversion of Na^+ and K^+ in the UW (high Na-UW) solution decreased reperfusion injuries and improved rat liver and kidney functions^[6,13,14]. With the isolated perfused rat liver model, we showed that PEG substitution

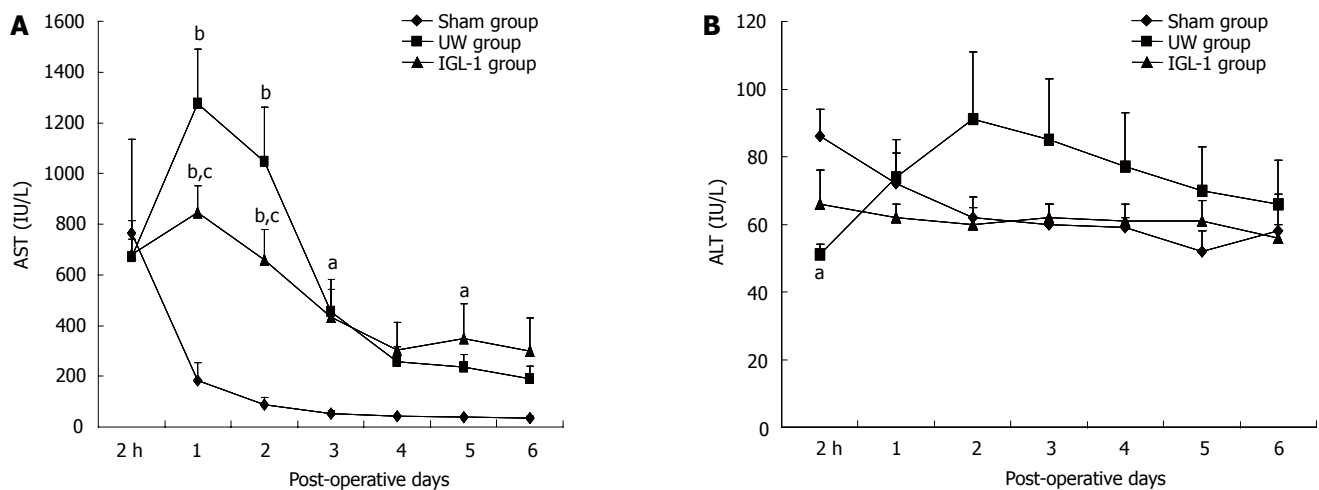


Figure 3 Serum aspartate aminotransferase (A) and alanine aminotransferase (B) activities after liver transplantation. Data are expressed as mean \pm SE. ^a $P < 0.05$ vs sham; ^b $P < 0.01$ vs sham; ^c $P < 0.05$ vs UW.

for HES in high Na-UW solution (IGL-1) was able to reduce parenchymal and non-parenchymal cell damage^[8]. In the autotransplanted pig model, kidneys preserved in IGL-1 solution showed a better function and a significant reduction of MHC class II expression, cellular apoptosis and interstitial fibrosis compared to the kidneys preserved in UW liquid^[9]. In a preliminary clinical study, we found an important improvement in creatinine clearance and a reduction in cellular apoptosis when IGL-1 was used^[10].

It is well known that porcine livers are very sensitive to cold ischemia. In the pig liver transplantation model and after 12 h of cold ischemia, it was reported that all animals died within 24 h of reperfusion, regardless of the cold storage solution used^[15]. That is why we preserved livers for 7 h in order to optimize the animal viability.

There is strong evidence that the period of rewarming ischemia after cold preservation and before reperfusion increases hepatocellular injury and affects graft outcome. Devlin *et al.*^[16] examined liver enzyme (AST, LDH, purine nucleoside phosphorylase and CK-BB) activities present in the organ preservation solution washed out from the graft at the end of ischemic storage prior to reperfusion. They found a correlation between AST activity level and early post-operative graft viability^[16], thereby suggesting that AST is a discriminative marker for post-transplant graft function. In addition, preservation-reperfusion injury is characterized biochemically by high serum AST levels in the early post-operative period^[17]. In our hands, a statistical difference in AST levels was found between the use of IGL-1 and UW preservation solutions in effluent preservation fluid during graft rinsing. The same results were also observed at 1-2 post-operative days. Although the results of ALT were not significantly different between the three experimental groups, we could notice the absence of any peak after the use of IGL-1. Our biochemical results suggest that IGL-1 preserves parenchymal architecture after cold storage. This is confirmed by the histological study where few necrotic regions were observed in livers cold stored in IGL-1.

Numerous previous studies demonstrated the

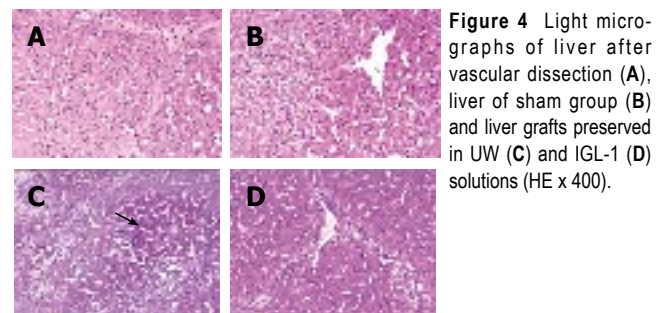


Figure 4 Light micrographs of liver after vascular dissection (A), liver of sham group (B) and liver grafts preserved in UW (C) and IGL-1 (D) solutions (HE x 400).

susceptibility and functional consequences of sinusoidal cells to hypothermic and reperfusion injury. Maintenance of endothelial cells integrity and viability is, therefore, of primary importance during organ transplantation. During the last few years, preservation injury and especially the endothelial cells injury occurring during liver preservation have been described to be due to apoptosis^[18-20]. This process is an early event after reperfusion, since it was observed that 3 h after rewarming, almost all cultured liver endothelial cells lost viability^[21]. One of the common parameters used in the assessment of sinusoidal cell viability is the release of CK-BB isoenzyme in the blood^[12]. Our results revealed that UW does not offer any protection against endothelial cell injury. In comparison to livers preserved in UW solution, livers preserved in IGL-1 released markedly less CK-BB after 2 h of reperfusion. Thus, IGL-1 seems to better protect non-parenchymal cells than UW.

During the last few years, several preservation solutions have been developed and studies comparing these solutions to UW have been carried out. Histidine-tryptophan-ketoglutarate (HTK) preservation solution, designed as a cardioplegia liquid, is now used routinely by many centers of abdominal organs preservation. Recent data suggested HTK to be an effective preservation solution of livers^[22]. Interestingly, HTK offered a powerful ability to preserve endothelial structure and function during warm ischemia^[23].

During storage, the preservation solution is in direct contact with the vascular endothelial bed. Injured endothelial cells are potent producers of cytokines and adhesion molecules that directly facilitate graft invasion by humoral and cellular components of inflammation^[24]. These mediators increase damage caused by ischemia or reperfusion and promote acute rejection and chronic rejection. It is reported that HES in UW enhances red blood cell aggregation^[4], leading to microvessel occlusive events and inflammation. In contrast, it is described that PEG in UW reduces the inflammatory injury due to cold ischemia-reperfusion in an autotransplanted pig kidney model^[25]. Our histological results showed no significant difference for inflammation between the two preserved groups.

In conclusion, data reported here indicate that IGL-1 efficiently preserves parenchymal and non-parenchymal cells associated with orthotopic liver transplantation. Despite the fact that the UW solution is the most commonly used cold storage liquid, HES and K⁺ limit its performances. IGL-1 (a PEG high-Na UW solution) could represent a valid alternative in organ preservation.

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Kinase domain insert containing receptor promoter controlled suicide gene system selectively kills human umbilical vein endothelial cells

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Abstract

AIM: To study the selective killing of human umbilical vein endothelial cells (HUVECs) by a double suicide gene under the regulation of a kinase domain insert containing receptor (KDR) promoter and mediated by an adenoviral gene vector.

METHODS: Human KDR promoter was cloned by polymerase chain reaction (PCR), and two recombinant adenoviral plasmids pAdKDR-CDglyTK, pAdCMV-CDglyTK were constructed according to a two-step transformation protocol. These two newly constructed plasmids were then transfected into 293 packaging cells to grow adenovirus, which were further multiplied and purified. HUVECs and LoVo cells were infected with either of the two resultant recombinant adenoviruses (AdKDR-CDglyTK and AdCMV-CDglyTK) respectively, and the infection rates were estimated by detection of green fluorescent protein (GFP) expression. Infected cells were cultured in culture media containing different concentrations of 5-fluorocytosine (5-FC) and ganciclovir (GCV), and the killing effects were measured.

RESULTS: The two recombinant adenoviral plasmids pAdKDR-CDglyTK, pAdCMV-CDglyTK were successfully

constructed and transfected into 293 cells. The resultant recombinant adenoviruses infected cells caused similar infection rates; and the infected cells exhibited different sensitivity to the prodrugs: HUVECs infected with AdCMV-CDglyTK and LoVo cells infected with AdCMV-CDglyTK were highly sensitive to the prodrugs, and HUVECs infected with AdKDR-CDglyTK were similarly sensitive but significantly more sensitive than the LoVo cells infected with AdKDR-CDglyTK ($P < 0.001$).

CONCLUSION: Selective killing of HUVECs may be achieved by gene transfer of double suicide gene under the regulation of the KDR promoter. This finding may provide an optional way to target gene therapy of malignant tumors by abrogation of tumor blood vessels.

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Key words: Human umbilical vein endothelial cells; Double suicide gene system; Targeted killing

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INTRODUCTION

Gene therapy is a novel technology leading to improved treatments of some types of cancer^[1-3]. On the other hand, anti-angiogenic therapy has also been proven to be a rational approach in the treatment of solid tumors also^[4-9]. Selective delivery of the suicide gene into tumor endothelial cells and target expression of it to abrogate tumor vasculature may be a good way for tumor therapy.

A suicide gene is a gene encoding an enzyme that converts nontoxic prodrugs into toxic forms. The herpes simplex virus-thymidine kinase (TK) gene is one of the most widely studied suicide genes^[10], and the *E. coli* cytosine deaminase (CD) gene is another widely studied one^[11-13]. However, results of numerous investigations aimed at eradicating tumors employing either CD or TK demonstrate their limitations. Whether the limitations

stem from unfavorable pharmacokinetics, loss of transgene expression or biochemical resistance is not clear. Nevertheless, fusion gene of CD and TK shows exciting superiority in many studies^[14-17].

The adenovirus vector system has many advantages^[18-20]. First, adenovirus vectors can be prepared at much higher titers than retroviral vectors and have a high efficiency of gene transfer regardless of the proliferative states of the tissues whereas retroviral vectors insert their genes only into dividing cells. Second, adenovirus genomes usually do not integrate into the host cell chromosome, and the level of therapeutic gene expression is very high. Human umbilical vein endothelial cells (HUVECs) are primary cells that can be used to investigate the mechanisms underlying the role of endothelial cells^[21]. The kinase domain insert containing the receptor (KDR) gene is strictly expressed only in vascular endothelium cells. The activity of the KDR promoter in endothelial cells is similar to that of the potent SV40 promoter/enhancer and that this high level activity is specific to endothelial cells^[22].

We constructed two recombinant adenoviruses to transfer the double suicide gene under the KDR promoter or the CMV promoter into HUVECs respectively, and LoVo cells (a cell system of colon carcinoma) were used as the control. Prodrug sensitive experiments were performed to evaluate the killing effect of the fusion double suicide gene under the regulation of the KDR promoter, mediated by the adenovirus vector on HUVEC cells.

MATERIALS AND METHODS

Materials

Shutter plasmid pAdtrack, pAdtrack-CMV, adenoviral backbone plasmid pAdEasy-1 and *E. coli* BJ5183 were provided by Dr. Belt Vogelstein at Johns Hopkins Oncology Center, Howard Hughes Institute of Medicine. pMD18-T vector was purchased from TaKaRa Biotechnology (Dalian) Co. Ltd. 293 cells, and HUVECs were obtained from American Type Culture Collection (ATCC). All sorts of exonuclease, T4 DNA ligase, and Taq DNA polymerase were purchased from New England Biolabs Co. DMEM, fetal bovine serum (FBS), transfection reagents, and LipofectAMINE2000 were products of Gibco Co. Primers of KDR promoter gene, CD gene, TK gene were synthesized and sequenced by Sangon Biotechnology (Shanghai) Co., Ltd.

KDR promoter sequence and CDglyTK sequence

KDR promoter genes (including the minimus core of the gene sequence -226~+268) were generated by PCR using human blood genome as the template (the upstream primer sequence: 5'-GGAAGATCTAGTTGCTCAGC-GCCCGTTAC-3', the downstream primer sequence: 5'-CCCAAGCTTGGCGAAATGCCAGAACTCG-3'). The resulting fragment containing Bgl at the 5' end, and HindIII restriction sites at the 3' end respectively were linked to pMD18-T vector to generate pMD-18KDR. Another two PCR protocols were performed to generate CD and TK gene sequences (the upstream primer sequence of CD: 5'-AAGCTTAGGCTAGCAATGTC-GAATAACGCT-3', the downstream primer sequence: 5'

-GGATCCTCCACGTTTGTAAATCGATGGCTTC-3'; the upstream primer sequence of TK: 5'-GGATCCG-GCGGGGGCGGTGGAGGAGGGGGTATGGCTTC-GTAC-3', the downstream primer sequence of TK: 5'-TCTAGATTAGTTAGCCTCCCCCATCTC-3'. The chromosome DNA of *E. coli* JM109 was used as template for CD gene amplification, and plasmid pREP8-TK for TK gene). According to the protocols, the initiation codon of the resulting CD gene was converted to ATG, termination codon of TAG to GGA which is to code for glycine, and its 5' end was to have BglII, and 3' end to have Hind III restriction sites respectively. The two amplified fragments were subcloned into pcDNA3 vectors to generate pcDNA3-CDglyTK.

Transfer plasmids construction

KDRs were removed from pMD-18KDR by BglII, Hind III and subcloned into pcDNA3-CDglyTK to generate pcDNA3-KDR-CDglyTK, which was digested with BglII and PvuII to get KDR-CDglyTK fragment. The resulting KDR-CDglyTK fragments were subcloned into pAdtrack to generate pAdtrackKDR-CDglyTK. CDglyTK fragment from pcDNA3-CDglyTK (HindIII, XbaI) was subcloned into pAdtrackCMV to construct pAdtrackCMV-CDglyTK.

Construction and identification of recombinant adenovirus vector plasmid

pAdEasy-1 plasmid was transformed into *E. coli* BJ5183, followed by growing of transformants on LB agar plates containing ampicillin and streptomycin. The transformed bacterium was named "AdEasy-1 bacteria".

pAdtrackKDR-CDglyTK was linearized and transformed into AdEasy-1 bacteria. Transformants were selected on LB agar plates containing 25 µg/mL kanamycin. Plasmid DNA was prepared from individual colonies, and agarose gel electrophoresis was performed. The correct recombinant pAdKDR-CDglyTK could be clearly identified by size, as only the 11.2-kb pAdtrackKDR-CDglyTK plasmid and the 37-kb pAdKDR-CDglyTK were selectable by kanamycin resistance.

The same protocol was performed to transfer pAdtrackCMV-CDglyTK into AdEasy-1 bacteria to recombine with pAdCMV-CDglyTK.

Propagation, purification, titer determination and identification of the recombinant adenovirus

Both pAdKDR-CDglyTK and pAdtrackCMV-CDglyTK were transferred into 293 cells mediated by LipofectAMINE2000 vector. Their further propagation was visualized under fluorescence microscope by GFP expression of transgene, and ultracentrifugation in CsCl gradient was performed to purify the viruses, and the titration of AdKDR-CDglyTK and AdtrackCMV-CDglyTK was measured by plaque formation assay.

The resultant recombinant viruses were boiled to be used as templates. PCR protocols were performed either to ensure the presence of CDglyTK gene in the viruses, in which upstream primer sequence of CD and downstream primer sequence of TK were used, or to ensure the

presence of the KDR promoter gene in AdKDR-CDglyTK. Primers of KDR promoter were used.

Cell culture and virus infection rate

HUVECs and LoVo cells, obtained from ATCC, were maintained in calorstart of 37°C, with 5% CO₂ and in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells (2×10^5 /well, 6-well plate) were infected with AdKDR-CDglyTK or AdCMV-CDglyTK at different multiplicity of infection (MOI). Percentages of cells expressing GFP were counted under fluorescence microscope within 3 d.

Prodrug sensitivity assays

HUVECs or LoVo cells (1×10^4 cells/well, 96-well plate, inoculated one day before) were infected with AdKDR-CDglyTK or AdCMV-CDglyTK at MOI of 100, 16 h later. The medium was removed and replaced with fresh medium with different concentrations of 5-FC and/or GCV. Cells were cultured in the presence of prodrugs for 72 h. Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's protocol.

Statistical analysis

Statistical analyses were made by ANOVA and LSD test. The values were calculated as means \pm SD. $P < 0.05$ was considered statistically significant.

RESULTS

Amplification or construction of gene fragments

The products of PCR amplification or constructed KDR promoter gene, CD and TK genes and CDglyTK gene were sequenced and verified by Sangon Biotechnology (Shanghai) Co., Ltd.

Recombinant viral plasmids

Newly constructed pAdtrackKDR-CDglyTK and pAdtrackCMV-CDglyTK were identified by restriction analysis and transferred into AdEasier-1 bacteria. Recombinant bacteria were selected by kanamycin. Figure 1 is the map of plasmids from 10 clones of transferred bacteria selected by kanamycin. Nine of them were correctly recombined, with a correction rate of 90% (9/10).

Recombinant viruses

Three days after transferring pAdKDR-CDglyTK and pAdCMV-CDglyTK into 293 cells, we found that most transferred 293 cells expressed GFP (Figure 2). The titer of the purified virus after being sufficiently propagated was 3.5×10^{12} pfu/L. No difference between AdKDR-CDglyTK and AdCMV-CDglyTK was observed.

The products of the PCR using the recombinant viruses as templates were proven to be correct by size comparison (Figure 3), with KDR promoter of 580-bp and CdglyTK of 2.5-kb.

Infection rate of viruses

HUVECs and LoVo cells infected with AdKDR-CDglyTK

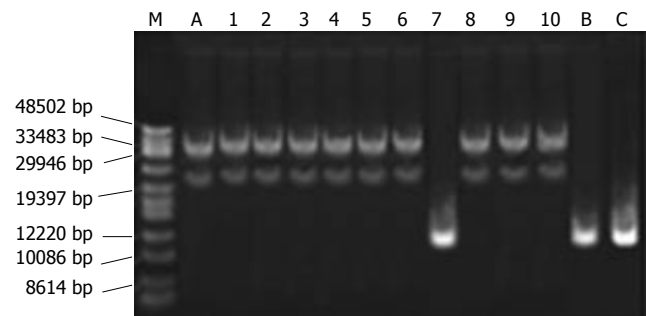


Figure 1 Selection of correct recombinants. M: Lambda mix marker, 19 (Fermentas Co.); A: pAdEasy-1 DNA; B: pAdtrackKDR-CDglyTK; C: pAdtrackCMV-CDglyTK; lanes 1-5: Plasmids of pAdtrackKDR-CDglyTK transferred AdEasy-1 bacterium; lanes 6-10: Plasmids of pAdtrackCMV-CDglyTK transferred AdEasy-1 bacterium.

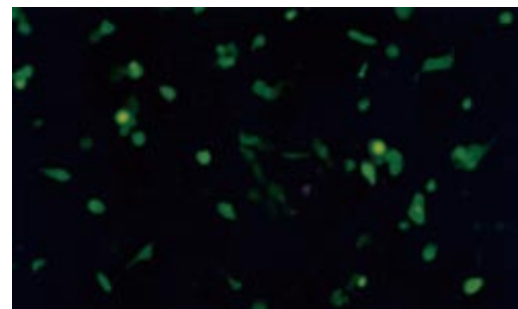


Figure 2 Recombinant viruses propagating in 293 cells. GFP expression was visualized by fluorescence microscopy 3 d after transfer of recombinant plasmids into 293 cells.

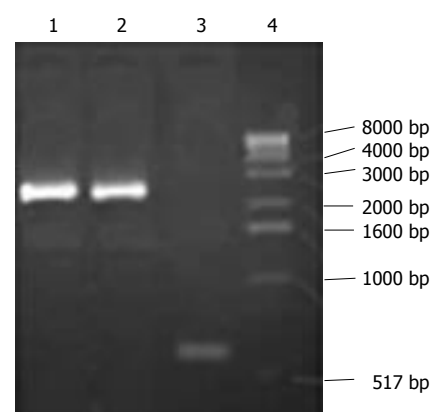


Figure 3 PCR amplification of CDglyTK and KDR promoter gene from the recombinant adenoviruses DNA. 1: PCR products of AdCMV-CDglyTK DNA using the upstream and downstream primers of CDglyTK gene; 2: PCR products of AdKDR-CDglyTK DNA using the upstream and downstream primers of CDglyTK gene; 3: PCR products of AdKDR-CDglyTK DNA using the upstream and downstream primers of KDR promoter gene; 4: 1 kb DNA ladder (products of Dingguo Biotechnology Development Center Co., Ltd.)

or AdCMV-CDglyTK at different MOI were observed 3 d post-infection (Figure 4). It was indicated that cell infection rate increased with the increase of MOI of the virus: when MOI = 1, only a few cells expressed GFP; when MOI = 100, most cells expressed GFP; when MOI = 200, almost all cells expressed GFP. There was no difference between the two recombinant viruses in infecting HUVECs and LoVo cells.

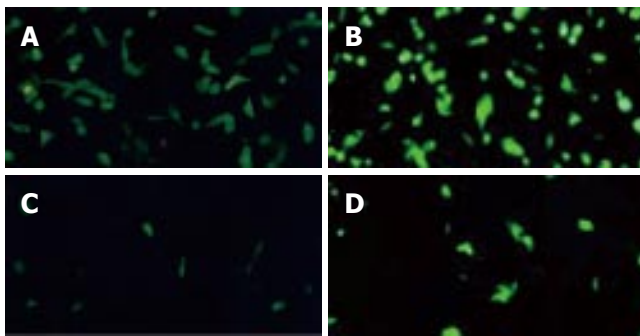


Figure 4 The recombinant viruses infected cells and transgene expression. **A, C:** GFP expression of HUVECs 5 d after infected with AdKDR-CDglyTK at MOI = 100 and MOI = 1; **B, D:** GFP expression of LoVo cells 5 d after infected with AdKDR-CDglyTK at MOI = 100 and MOI = 1.

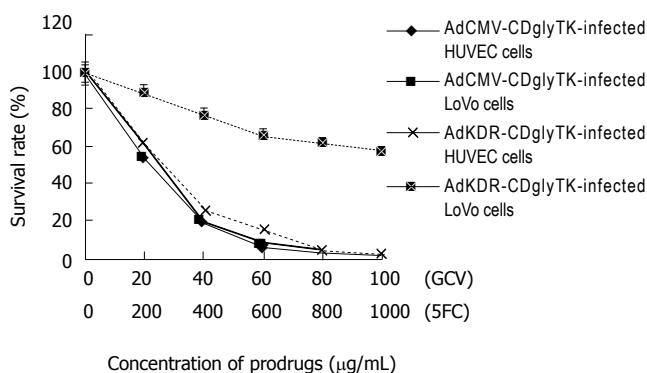


Figure 5 Killing effect of prodrugs to transgenic cells.

Killing effect of prodrugs

HUVECs and LoVo cells infected with AdKDR-CDglyTK or AdCMV-CDglyTK at MOI of 100 were maintained in culture medium of different concentration of GCV and 5-FC for 3 d, and the survival rates were measured (Figure 5). Both AdCMV-CDglyTK-infected HUVECs and AdCMV-CDglyTK-infected LoVo cells were highly sensitive to the prodrugs. More than 80% of both transgene cells were killed when they were treated with 40 µg/mL GCV + 400 µg/mL 5-FC; almost all of both types of cells were killed when treated with 100 µg/mL GCV + 1000 µg/mL 5-FC. The sensitivities of these two transgenic cells were of no much difference ($P = 0.518$). AdKDR-CDglyTK-infected HUVECs were highly sensitive to the prodrugs, similarly ($P > 0.2$); however, AdKDR-CDglyTK-infected LoVo cells were far less sensitive to the prodrugs ($P < 0.001$). There was still 57.2% survival of cells 3 d after treatment with 100 µg/mL GCV + 1000 µg/mL 5-FC.

DISCUSSION

A blood supply is required for a tumor to progress in excess of 1-2 mm³[23,24]. Recent data suggest that tumor cells can co-opt surrounding vasculature from an even smaller size in certain tumor types[25]. The expanding growth of a primary tumor is associated with neo-angiogenesis and vessel maturation of the tumor.

Abrogation of tumor blood vessel should lead to the eradication or suppression of solid tumor. Moreover, Folkman proposed that the prevention of tumor vascularization essentially stops neoplastic invasion, which may lead to the ablation of metastatic disease[26]. The rapid growth of a tumor results in hypoxia[27] which induces the production of pro-angiogenic factors such as vascular endothelial growth factor (VEGF)[28]. VEGF is a potent and specific mitogen for endothelial cells. It plays a major role in angiogenesis and vasculogenesis. KDR and Flt-1 are the two receptor tyrosine kinases that regulate the actions of VEGF and are expressed in endothelial cells. KDR is critically involved in the regulation of angiogenesis, both in the developing and adult animals[29]. The vascular endothelial cell is renewed at a low speed in normal conditions, and its KDR expression level is very low, while tumor vascular endothelial cells proliferate quickly and with a KDR expression level 500 times higher than that of vascular endothelial cells of normal tissues[30]. Therefore, it is possible to realize targeted expression of therapeutic genes in tumor vascular endothelial cells by transcriptional regulation of the KDR promoter, thus markedly reducing the toxicity and side effects of gene therapy targeting vascular endothelial cells of tumors.

In our experiments, we constructed two replication-incompetent recombinant adenoviral vectors with the AdEasy system in a “two-step transformation protocol” to transfer the KDR-promoter-controlled/CMV-promoter-controlled double suicide gene into HUVEC/LoVo cells. The results indicated that “two-step transformation protocol” is a convenient and efficient way to generate adenoviral vectors. The resultant adenoviruses infected HUVECs with a great efficiency and the transgenes were efficiently expressed, which was delineated by the reporter gene GFP as it was expressed in almost all HUVECs when they were infected with the virus at MOI of 200. The subsequent prodrug sensitivity experiments demonstrated that this high level expression was strong enough for cell killing *in vitro*. The prodrug sensitivity experiment also demonstrated that CMV-promoter-controlled double suicide gene/prodrugs system unselectively killed both HUVECs and LoVo cells; however, the KDR-promoter-controlled double suicide gene/prodrugs system displayed a targeted killing effect on HUVECs: treated with 40 µg/mL GCV + 400 µg/mL 5-FC, 74.8% KDR-CDglyTK-transferred HUVECs were killed while 77.6% KDR-CDglyTK-transferred LoVo cells were alive; treated with 100 µg/mL GCV + 1000 µg/mL 5-FC, 98.5% KDR-CDglyTK-transferred HUVECs were killed while the killing rate of KDR-CDglyTK-transferred LoVo cells was 42.8%. All these come to an exciting conclusion that: the KDR-promoter-controlled double suicide gene/prodrugs system bears a selectively killing effect on HUVEC. This finding may provide an optional way for targeting gene therapy of malignant disease by abrogation of tumor blood vessels.

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CLINICAL RESEARCH

Ursodeoxycholic acid improves gastrointestinal motility defects in gallstone patients

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Abstract

AIM: To simultaneously evaluate the presence of defects in gallbladder and gastric emptying, as well as in intestinal transit in gallstone patients (GS) and the effect of chronic ursodeoxycholic acid (UDCA) administration on these parameters and on serum bile acids and clinical outcome in GS and controls (CTR).

METHODS: After a standard liquid test meal, gallbladder and gastric emptying (by ultrasound), oroileal transit time (OITT) (by an immunoenzymatic technique) and serum bile acids (by HPLC) were evaluated before and after 3 mo of UDCA (12 mg/kg bw/d) or placebo administration in 10 symptomatic GS and 10 matched healthy CTR.

RESULTS: OITT was longer in GS than in CTR ($P < 0.0001$); UDCA significantly reduced OITT in GS ($P < 0.0001$), but not in CTR. GS had longer gastric half-emptying time ($t_{1/2}$) than CTR ($P < 0.0044$) at baseline; after UDCA, $t_{1/2}$ significantly decreased ($P < 0.006$) in GS but not in CTR. Placebo administration had no effect on gastric emptying and intestinal transit in both GS and CTR.

CONCLUSION: The gallstone patient has simultaneous multiple impairments of gallbladder and gastric emptying, as well as of intestinal transit. UDCA administration restores these defects in GS, without any effect in CTR. These results confirm the pathogenetic role of gastrointestinal motility in gallstone disease and suggest an additional mechanism of action for UDCA in reducing bile cholesterol supersaturation.

INTRODUCTION

Cholesterol gallstone disease is frequent, in fact its prevalence ranges from 10% to 20% in Western countries^[1]. Although most gallstone patients (GS) are asymptomatic^[1,2], a progressively increased rate of cholecystectomies has been reported^[3], thus confirming that gallstone disease is one of the major gastrointestinal problems throughout the Western world. Cholesterol gallstone pathogenesis is complex and multifactorial, involving both genetic defects and environmental factors^[4,5], all of which contribute to cholesterol-supersaturated bile, rapid nucleation time of cholesterol microcrystals (as a result of excess promoters and/or a deficiency of crystallization inhibitors), and impaired gallbladder motility. However in GS not only is gallbladder motility defective^[6], but it has been recently documented^[7-9] that also intestinal transit time and gastric emptying are delayed. However, no study has simultaneously evaluated gallbladder and gastric emptying and intestinal transit in GS.

Ursodeoxycholic acid (UDCA) is a drug known to be able to reduce bile cholesterol supersaturation and to dissolve cholesterol gallstones^[10], without significant side effects^[11]. Reduction in bile cholesterol supersaturation is reached, according to some^[12,13] but not to others^[14,15], mainly through a reduction in intestinal cholesterol absorption and by formation of a liquid crystalline phase^[16]. Furthermore, UDCA has been proposed in gallstone prevention in subset populations, i.e. obese patients during weight loss^[17] and more recently, it has been shown that chronic, long-term UDCA administration reduces biliary pain in GS^[18]. Conflicting results exist regarding the effect of UDCA on gallbladder motility, since fasting and residual gallbladder volumes were found to be increased while gallbladder emptying has been

Table 1 Characteristics of gallstone patients (GS) and healthy controls

Characteristics	GS (n = 10)	Controls (n = 10)	P
Gender			
Male	4	4	
Female	6	6	
Age (yr)			
Mean \pm SE	49.5 \pm 2.3	50.5 \pm 1.4	NS
Range	30-63	28-61	
BMI (kg/m²)			
Mean \pm SE	23.0 \pm 0.8	22.8 \pm 0.4	NS
Range	21-28	20-29	
Biliary colic	10/10	0/10	
Gallstones			
n (single/multiple)	3/7		
Size (< 1.5 cm)	10/10		

NS: Not significant.

found to be either decreased or unmodified^[6,19]. Moreover, no definitive data is available on the effect of chronic UDCA administration on gastrointestinal (GI) motility in man.

In a preliminary study aimed at simultaneously evaluating gallbladder and gastric emptying and intestinal transit before and after UDCA treatment in GS and healthy subjects, we found^[20] that GS presented impaired gallbladder and gastric emptying and delayed intestinal transit and that UDCA administration improved these defects in GS without inducing any significant effect in healthy subjects.

Since these observed effects of UDCA administration could have been biased by the lack of a placebo arm in the study protocol, we performed a new placebo-controlled study aimed at evaluating the effect of chronic administration of UDCA on gastric and gallbladder emptying and intestinal transit in GS and controls.

MATERIALS AND METHODS

Study design

This single blinded, placebo controlled study was designed to evaluate the effects of UDCA, or placebo, on meal stimulated gallbladder and gastric emptying, small intestinal transit and serum bile acid pattern in GS and healthy controls (CTR). All individuals were assigned to UDCA for the first treatment period of the study lasting 3 mo, then after an interim 15 d washout period, placebo was administered for additional 3 mo. Patients and controls were unaware of the study design, while ultrasonographers were unaware of the results of biochemical analyses (serum bile acid determination, intestinal transit time), and biochemical investigators were unaware of the results of ultrasonographic investigations.

The study design was in accordance with the ethical guidelines of the Declaration of Helsinki and approved by the Local Ethical Committee. Written informed consent was obtained from all subjects.

Study population

The studied population was comprised of 10 consecutive GS (4 males, 6 females, mean age 49.5 \pm 2.2 years, range

Table 2 Baseline biochemical parameters in gallstone patients (GS) and controls (mean \pm SD)

Biochemical parameter	GS (n = 10)	Controls (n = 10)	P
Serum creatinine (mg/dL)	0.98 \pm 0.25	0.96 \pm 0.21	NS
Blood nitrogen (mg/dL)	39.4 \pm 13.0	37.7 \pm 10.6	NS
AST (IU/l)	23.2 \pm 15.0	21.3 \pm 12.7	NS
ALT (IU/l)	23.9 \pm 14.6	21.8 \pm 11.6	NS
AP (IU/l)	147.3 \pm 63.2	150 \pm 60.5	NS
γ GT (IU/l)	28.0 \pm 18.0	30.2 \pm 15.8	NS
Total proteins (g/dL)	6.8 \pm 0.57	6.9 \pm 0.58	NS
Total cholesterol (mg/dL)	185.0 \pm 38.5	178.4 \pm 35	NS
Fasting triglycerides (mg/dL)	141.7 \pm 30.8	138.5 \pm 27.2	NS
Fasting glucose (mg/dL)	96.8 \pm 12.0	95.3 \pm 11.4	NS

NS: Not significant.

30-63; mean body mass index (BMI) 23.0 \pm 0.8 kg/m² and 10 matched CTR (4 males, 6 females, mean age 50.5 \pm 1.4 years, range 28-61; mean BMI 22.8 \pm 0.4 kg/m²). CTR were recruited from local staff members of our department. Characteristics of patients and controls are shown in Table 1, while in Table 2 the biochemical parameters are reported. All enrolled GS had cholesterol gallstones, as evaluated by oral cholecystography. They had a history of biliary pain, defined according to the previously identified criteria^[2], as a pain at the epigastrium or right hypochondrium, unrelated to meal ingestion, which does not disappear with bowel movements. No GS had past or present signs of complicated gallstone disease (i.e. acute cholecystitis, biliary obstruction or biliary pancreatitis). Furthermore, no GS had other GI and/or liver diseases or was taking drugs potentially affecting gastrointestinal function. All GS were suitable to bile acid dissolution therapy according to the indications of a recent Working Team Report^[21], i.e. radiolucent gallstones less than 1.5 cm in diameter, in functional gallbladder. All GS patients and controls had daily bowel evacuation and fertile female subjects were studied within the follicular phase to prevent any influence of hormones on GI motility behaviour.

Study protocol

Gallbladder and gastric emptying, oro-ileal transit time (OITT) and serum bile acids were assessed in all subjects at enrollment, after 3 mo administration of UDCA (*Deursil® Sanofi-Synthelabo, Paris-Cedex, France*) (12 mg/kg bw/d), after the wash-out period and at the end of placebo administration. Furthermore, the presence of biliary pain and/or dyspeptic symptoms (i.e. heartburn, vomiting, nausea, belching, bloating feeling after meals, intolerance to fatty or fried foods, epigastric discomfort, uncomfortable feeling at the right hypochondrium), were evaluated before and during treatment.

Gallbladder emptying

Gallbladder emptying was evaluated by a previously described ultrasonographic technique using the ellipsoid method^[6]. Both GS and control subjects were studied after an overnight fast. Gallbladder volume variations were measured in response to a standard liquid test meal (200 mL containing 375 kcal, 17 g fats, 10.4 g proteins, 10 g carbohydrates); measurements were performed before and

every 10 min after meal ingestion until 80% gallbladder refilling was reached. Results were expressed as fasting gallbladder volume (FV, in mL), given as the mean of four measurements obtained before meal intake; residual gallbladder volumes (RV, mL), given by the minimal gallbladder volume after postprandial gallbladder emptying, and percent gallbladder emptying (%E), given by: $(FV - RV / FV) \times 100$. All measurements were performed by two sonographers (AC, LS) and inter- and intra-observer variations less than 8% were documented.

Oro-ileal-transit time

OITT was evaluated as previously described^[8], using the tauroursodeoxycholic acid (TUDCA) load test and evaluating serum TUDCA appearance time. TUDCA was used since this bile acid is selectively and rapidly absorbed in the terminal ileum^[22]. After 12 h of fasting, each individual was given the standard liquid test meal to which 1.5 g of TUDCA was added. Blood samples were collected by peripheral venous catheter every 30 min for 7 h and then stored at -20°C until analyzed. OITT was evaluated measuring serum concentrations of TUDCA by means of an enzymatic immunoassay using a specific antibody against this bile acid^[23]. The assay fulfils all the requirements of precision and accuracy; the intra- and inter- assay precision, evaluated on six replicates of human serum samples spiked with three levels of TUDCA (0.001, 0.01, 0.1 $\mu\text{mol/L}$), presents a CV % below 8%. The accuracy of the method has been evaluated by comparison with the results obtained using a high-pressure-liquid chromatography -ES- mass spectrometry (HPLC-ES-MS) reference method^[24].

OITT was defined, as previously described^[8], as the time-interval between test meal administration and peak serum concentration of TUDCA.

Serum bile acid levels

Fasting serum bile acids were measured by HPLC-MS^[24]. In brief, serum samples (0.1 mL) were diluted with 3.5 mL of 0.1 mmol/L NaOH, incubated at 64°C for 30 min and submitted to a clean-up procedure by means of a conventional C18 reverse-phase extraction. The apparatus consisted of an analytical HPLC system (Alliance 2695 Waters, Milford, MA, USA) connected with a triple quadrupole mass spectrometer (Quattro LC, Micromass, Wythenshawe, UK), with electrospray interface. Individual bile acids were separated on a C18 analytical column (Ultrasphere XL, 3 μm , 70 mm \times 4.6 mm I.D., Beckman Instruments, Berkeley CA, USA) and analysed by electrospray mass spectrometry in the negative-ion acquisition mode following the procedure previously described^[24].

Gastric emptying

Gastric emptying was evaluated simultaneously with gallbladder motility, monitoring changes in the gastric antral area by an ultrasonographic technique^[9,25]. Gastric antral area variations were evaluated before and after administration of the same standard liquid test meal used to assess gallbladder motility. Results were expressed as

half-emptying time ($t_{1/2}$): the time, in minutes, to observe a 50% decrease in maximal antral area measured by linear regression analysis.

Statistical analyses

All results were analysed using the Statistical Package for Social Science (SPSS version 11.5). Unless otherwise stated, all results were expressed as mean \pm SE. Student's *t* test for paired and unpaired data was used to evaluate significant intra- or inter-group differences. A repeated measure ANOVA model was individually fitted for each of the following variables: gallbladder basal and residual volume, gallbladder emptying, intestinal transit time, gastric emptying, serum levels of cholic (CA), deoxycholic (DCA), lithocholic (LCA), ursodeoxycholic (UDCA) and chenodeoxycholic (CDCA) acids. Treatment (basal, UDCA and placebo) was considered as the within-subject factor and controls versus GS was considered as the between-subject factor.

Univariate and multivariate linear regression analyses were performed to evaluate the relationship between serum bile acid levels and gastric $t_{1/2}$, OITT, fasting gallbladder volume and gallbladder emptying. Each statistical test was conducted at the significance level of 0.05 (two-sided).

RESULTS

Clinical outcome

All enrolled individuals completed UDCA or placebo treatment and no adverse treatment-related events, and in particular stool frequency change, were recorded. At enrolment, all GS were symptomatic and dyspeptic symptoms were present in 4 out of 10 GS (40%). No significant difference was present between controls and GS in terms of frequency and severity of dyspeptic symptoms. Biliary pain disappeared during UDCA treatment in all GS, while during placebo administration 20% of patients had symptom recurrence. No significant change (reduction or increase) in the number or size of the gallstones was observed during the study period.

Serum bile acids

Serum bile acid profiles were different in GS and CTR (Figure 1) groups. Baseline serum DCA was significantly higher in GS than controls (DCA%: 25.7 ± 0.5 vs 16.7 ± 0.4 , $P < 0.05$); LCA was higher in GS than controls (LCA% = 2.6 ± 0.5 vs 1.1 ± 0.2), but not significantly, while CA and CDCA were significantly lower in GS than in CTR (CA%: 33.2 ± 1.1 vs 37.2 ± 0.6 ; $P < 0.02$; CDCA %: 37.8 ± 0.7 vs 43.7 ± 0.6 ; $P < 0.05$).

After UDCA administration, CA, DCA and CDCA significantly decreased both in GS and CTR ($P < 0.05$); UDCA significantly increased ($P < 0.05$), reaching up to 60% of total serum bile acids in both groups (Figure 1). After placebo administration, in both GS and CTR, serum bile acid proportion did not significantly change compared to baseline values.

Oro-ileal-transit time

At baseline, GS showed longer OITT compared to

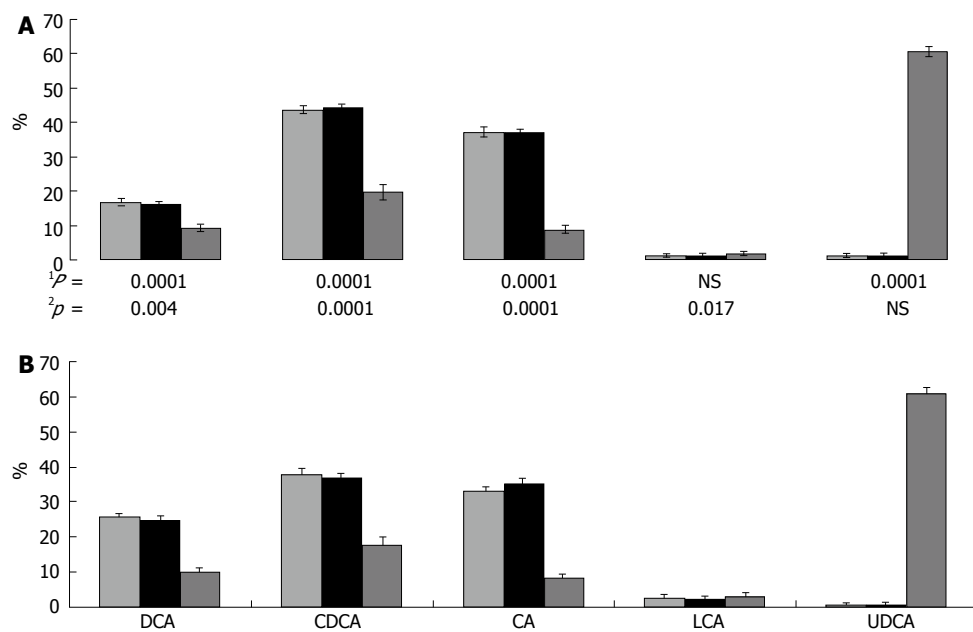
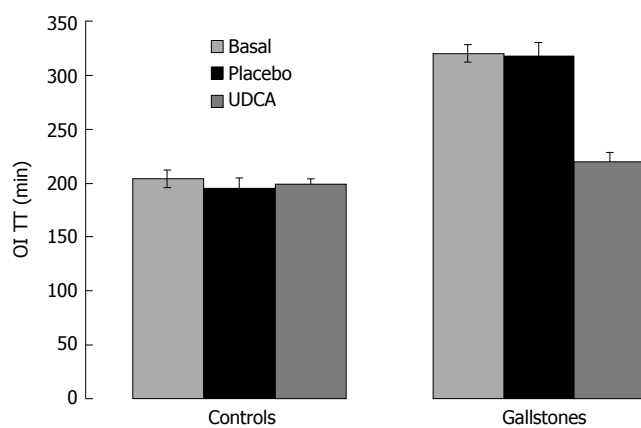


Figure 1 Serum bile acids concentrations (percent values) in gallstone patients (down) and controls (up), before (□) and after placebo (■) and ursodeoxycholic acid treatment (▨). Each bile acid concentration is expressed as percentages of total serum bile acids. DCA: Deoxycholic acid; CDCA: Chenodeoxycholic acid; CA: Cholic acid; LCA: Lithocholic acid; UDCA: Ursodeoxycholic acid.

¹P value by ANOVA from testing treatment comparison.

²P value by ANOVA from testing between controls and gallstone patients overall across all treatments; NS: Not significant.



ANOVA: $P = 0.0001$ from testing treatment comparison.

$P = 0.0001$ from testing between controls and gallstones patients overall across all treatments.

Figure 2 Oro-ileal transit time (OITT) in gallstone patients and healthy controls before (□) and after placebo (■) and ursodeoxycholic acid (UDCA) administration.

controls (GS: 320.0 ± 6.9 min *vs* CTR: 204.0 ± 7.2 min.; $P < 0.05$) (Figure 2). UDCA treatment significantly reduced OITT in GS patients ($P < 0.05$) but not in controls. At the end of treatment, OITT was no longer significantly different in GS and controls (219.5 ± 8.9 min and 198.0 ± 8.8 min, respectively). Placebo administration did not significantly modify OITT in both GS and controls.

Gallbladder emptying

At baseline, with respect to controls, GS showed significantly larger fasting gallbladder volume (GS: 23.5 ± 0.9 mL *vs* CTR: 18.8 ± 0.9 mL, $P < 0.05$) and residual gallbladder volume (GS: 8.5 ± 0.3 mL *vs* CTR: 4.7 ± 0.8 mL, $P < 0.05$) than controls; percent gallbladder emptying was significantly smaller in GS than in controls (63.8 ± 2.4 and 76.0 ± 2.3 , respectively, $P < 0.05$) (Table 3).

Table 3 Fasting and residual gallbladder volumes, and percent gallbladder emptying before and after ursodeoxycholic acid (UDCA) and placebo administration in gallstone patients ($n = 10$) and healthy controls ($n = 10$)

	Basal	UDCA	Placebo	P^2
Fasting Volume (mL):				
Controls	18.8 (0.9)	22.9 (0.6)	19.1 (0.9)	0.001
Gallstones	23.5 (0.9)	26.2 (0.5)	24.1 (0.9)	
P^1	0.0001			
Residual Volume (mL):				
Controls	4.7 (0.8)	6.4 (0.7)	4.9 (0.7)	0.003
Gallstones	8.5 (0.3)	11.0 (1.0)	8.5 (0.2)	
P^1	0.0001			
Gallbladder emptying (%)				
Controls	76 (2.3)	71.9 (3.2)	74.5 (2.2)	0.066
Gallstones	63.8 (2.4)	59.5 (3.3)	61.6 (2.2)	
P^1	0.001			

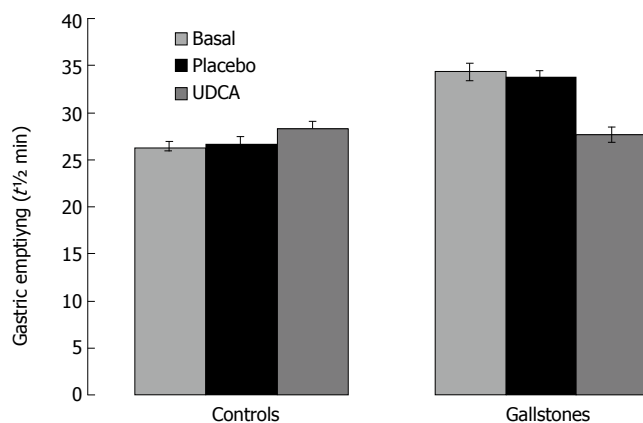
Repeated measures Analysis of Variance. ¹From testing between controls and gallstone patients overall across all treatment; ²For testing the treatment effect (basal, UDCA, placebo).

No significant effect was observed after placebo treatment in both GS and controls; after UDCA treatment, fasting and residual gallbladder volumes significantly increased both in GS and controls, while the percent of gallbladder emptying did not significantly change in both groups, as shown in Table 3. No difference was observed in fasting and gallbladder emptying between basal UDCA and basal placebo treatment.

Gastric emptying

At baseline half emptying time ($t_{1/2}$) was significantly longer in GS than in controls (34.3 ± 1.8 min and 26.4 ± 1.7 min, respectively, $P < 0.05$) (Figure 3).

Following UDCA, $t_{1/2}$ was significantly reduced only in GS, while no significant change was observed in controls; as a consequence, half-emptying time was similar in the



ANOVA: $P = 0.03$ from testing treatment comparison.

$P = 0.015$ from testing between controls and gallstones patients overall across all treatments.

Figure 3 Gastric half emptying-time ($t_{1/2}$, min) in gallstone patients and healthy controls before (□) and after placebo (■) and ursodeoxycholic acid (UDCA) (▨) administration.

two groups (GS: 27.6 ± 1.6 min, controls: 28.3 ± 1.6 min). Gastric $t_{1/2}$ did not change significantly after placebo in either GS or controls (Figure 3).

Relationship between serum bile acid, gallbladder and gastric emptying, and intestinal transit

At baseline, a direct linear correlation between serum DCA and OITT ($P = 0.0001$) and serum DCA and gastric $t_{1/2}$ ($P = 0.024$) was observed, while an inverse relationship was present for CA and OITT ($P = 0.001$) as well as for CDCA *vs* OITT ($P = 0.0001$) (Table 4). Furthermore, serum DCA percent concentrations directly related with fasting gallbladder volume ($P = 0.0007$) and inversely with percent gallbladder emptying ($P = 0.004$). At multivariate analysis, only DCA significantly related to OITT ($b = 12.1$, 95% CI = 6.2-17.9, $P = 0.0001$). The relationships found to be present at univariate analysis remained after placebo administration, but they disappeared at the end of UDCA treatment.

DISCUSSION

Data emerging from the present study, simultaneously evaluating the motor behaviour of the various GI segments in cholesterol gallstone patients and in healthy subjects, documented in GS the presence of multiple defects involving the gallbladder, the stomach and the small intestine. However, the most important aspect of this paper is that only chronic UDCA administration, and not placebo, significantly influenced gallbladder and gastric emptying, and intestinal transit. Furthermore, this effect is achieved only in GS, being UDCA ineffective in controls. In fact, only in GS did oro-ileal transit time and gastric emptying become faster, normal values being achieved at the end of the UDCA treatment period. All of these effects occurred with a significant improvement in clinical manifestations of the gallstone disease; in fact, most patients no longer experienced episodes of biliary pain during treatment with UDCA.

Table 4 Relationship between serum bile acids and intestinal transit time (OITT), gastric half emptying time ($t_{1/2}$ min) and gallbladder emptying (%) in gallstone patients ($n = 10$) and healthy controls ($n = 10$) (univariate analysis)

	OITT (min)		Gastric emptying ($t_{1/2}$, min)		Gallbladder emptying (%)	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
CA	-0.696	0.001	-0.118	0.62	0.44	0.05
DCA	0.887	0.0001	0.503	0.024	-0.617	0.004
CDCA	-0.728	0.0001	-0.547	0.012	0.594	0.006

CA: Cholic acid; DCA: Deoxycholic acid; CDCA: Chenodeoxycholic acid.

It is accepted that among the different events contributing to gallstone formation, motility defects could play an important role^[5]; however only gallbladder dysfunction has been extensively studied and confirmed evaluating both gallbladder volume variations by US and gallbladder contractility^[26]. In the present study, GS showed, when compared to controls, greater fasting and residual gallbladder volumes, and a decrease in percent gallbladder emptying, confirming previous observations from our experience^[6] and that of other groups^[19,27].

In GS a prolonged time of the migrating motor complex and an altered motilin release pattern have been demonstrated during the interprandial period, leading to an increased fasting gallbladder volume^[28]. The pathogenetic mechanism responsible for gallbladder hypocontractility has been related to an excessive accumulation of cholesterol molecules within the sarcolemma membrane of smooth muscle cells leading to stiffening of the cell membrane and to impairment in the agonist-receptor signal transduction and defective relaxation^[29,30]. Moreover, a role of mucosal inflammation and of inflammatory mediators induced by cholesterol accumulation has also been suggested to be present in the pathway of events leading to defective gallbladder contractility^[31].

In the present study GS patients showed longer OITT than controls, confirming previous observations from our^[8] and other laboratories^[7,27]. The pathogenetic mechanisms underlying this intestinal abnormality are not well understood, even if some hypotheses have been advanced.

The sluggish intestinal transit, together with gallbladder stasis, could contribute to the increase of DCA formation, allowing longer exposure of CA to intestinal bacteria^[32]. In fact we observed higher serum DCA concentrations in GS than in controls. However, this observation is in agreement with some authors^[7,8,27], but in contrast with others^[33,34]. Furthermore, we found in GS a direct linear correlation between serum DCA levels and OITT, and an inverse correlation between serum CA and OITT. As far as concerns the DCA mechanism of action on intestinal motility, it has been demonstrated in experimental animals that DCA directly delays OITT inducing a defective contractility of intestinal smooth muscle cells in response to cholecystokinin (CCK)^[35] or inhibits the pace-maker currents of interstitial cells of Cajal by activating ATP-sensitive K^+ channels through the production of PGE_2 ^[36]. However a cause-effect relationship is not proven.

In the present study, a direct correlation was demonstrated in GS between fasting gallbladder volume and serum DCA levels and an inverse correlation between percent gallbladder emptying and DCA serum levels. It is important to note that DCA also has an effect on gallbladder motility, since it has been demonstrated that it is able to induce both relaxation and defective contraction of gallbladder smooth muscle, probably involving intramural neurons^[37].

As far as gastric motility is concerned, we found a delay in half emptying-time in GS compared to controls. Although this result was obtained using a liquid meal, and not a solid or semi-solid meal, which is considered as the gold standard, it was obtained in the same subject before and after treatments. However, the liquid meal has just been previously used in evaluating gastric emptying by others^[9]. Even for this defect the pathogenetic mechanism is not clear, although a detrimental effect of DCA on smooth muscle cells could be proposed^[38] and it has been speculated that this impaired motility could be related with hormonal alterations, in particular of neurotensin, as suggested for gallbladder^[39] and gastric motility^[40].

Apart from the speculation based on a pathogenetic role for DCA, other hypotheses could be formulated to explain the mechanisms underlying the diffuse motility defects present in GS. Bile cholesterol supersaturation (e.g. the main and indispensable pathogenetic factor in GS formation)^[10], in itself, could play a role. In experimental studies, a cholesterol-rich diet was associated with impaired small intestinal smooth muscle contractility and prolonged small intestinal transit time^[41]. Cholesterol accumulation in the intestinal smooth muscle cells could modify membrane fluidity, which, in turn, affects the activity of membrane regulatory proteins, such as receptors and ion channels^[42,43]. These proteins control electrical excitability and influence the periodicity of events, such as migrating motor complex^[28]. Alternatively it has been recently speculated in an experimental study^[44] that a key event underlying cholesterol gallstone pathogenesis could be a CCK-r deficiency, that could provoke intestinal and gallbladder motility dysfunction, thus allowing an excessive cholesterol absorption by both organs and inducing a vicious cycle.

The most important result emerging from the present study concerns the effect of UDCA on gastric and gallbladder emptying and intestinal transit. UDCA significantly improved gastric emptying and OITT, but only in GS since no significant change occurred in controls, while placebo administration had no effect in both GS and controls.

This result could be partially biased by the study design, which was a single blinded, placebo controlled one. However, since both patients and controls were unaware of the study design, we believe to have correctly detected the different effects of UDCA and placebo.

This result confirms those of our previous, uncontrolled study^[20], stresses the effects of UDCA on gallbladder and gastric emptying and intestinal transit. These effects could be due to a protective action of UDCA^[35,45] towards the damaging action of DCA (the relative proportion of which we found to be significantly diminished) and/or to an abil-

ity to reduce cholesterol bile supersaturation by influencing cholesterol bile secretion^[10] and/or intestinal cholesterol absorption^[13]. The reduction of cholesterol bile saturation could improve intestinal contractility by a mechanism similar to that observed at the level of gallbladder smooth muscle cells^[46].

As far as gallbladder motility in GS is concerned, while there is a general agreement regarding the effect of UDCA in increasing fasting and residual gallbladder volumes, that on gallbladder emptying is controversial. In fact either a decreased or unmodified emptying was observed in *in vivo* studies^[6,19].

As far as the different effects of UDCA on gastric emptying and intestinal transit time and on gallbladder emptying is concerned, we can only suggest some hypotheses. The damaging effect of lithogenic bile on gallbladder could be more pronounced with respect to other organs and, consequently, more time is probably needed to obtain a positive effect.

All these documented effects of UDCA on gastrointestinal motility suggest for this bile acid an additional mechanism of action^[10] in inducing bile cholesterol under saturation. In particular, the reduction in intestinal transit time could influence intestinal cholesterol absorption, as suggested by the experimental study performed by Wang *et al*^[44].

A further effect of UDCA administration was the reduction in biliary pain rate. In fact, all our GS patients were symptomatic at enrollment, but none of them reported further episodes of biliary pain during UDCA treatment. On the contrary, during placebo treatment, a 20% recurrence of biliary pain rate was observed. Although these results were obtained in a small population and after a relatively short period, it seems important from a clinical point of view. Furthermore, a positive effect of UDCA on symptom frequency has been reported by us in a randomized study comparing UDCA and CDCA as gallstone dissolving agents^[47] and more recently by others on a larger population and for a longer period of time^[18]. Different hypotheses have been advanced to explain the effect of UDCA on biliary pain frequency: dissolution of microlithiasis or crystals^[18], increased bile wash-out within the gallbladder^[48] and reduction of bile viscosity and sedimentable fractions^[49].

In conclusion, this study documents that gallstone patients do not have a single defect (defective gallbladder emptying), but simultaneous, multiple defects (delayed gastric emptying and intestinal transit). Chronic UDCA administration influences these parameters only in gallstone patients, but not in controls; thus restoring impaired OITT and gastric emptying, and suggesting an additional mechanism of action for UDCA in reducing bile cholesterol saturation. Furthermore UDCA improves clinical outcomes in gallstone patients. Further experimental and clinical studies are needed to better elucidate the mechanisms of action of UDCA on gastrointestinal motility. Furthermore, it will be important to evaluate the presence of gastrointestinal motility defects also in patients at high risk of gallstone development (obese subjects, patients with insulin-resistance syndrome,

pregnant women, etc), to confirm their pathogenetic role in GS formation and to identify a possible relationship with genetic and/or environmental factors.

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CLINICAL RESEARCH

Alteration of peripheral blood lymphocyte subsets in acute pancreatitis

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Abstract

AIM: To evaluate peripheral blood lymphocyte subsets in patients with acute pancreatitis (AP).

METHODS: Twenty patients with mild AP (M-AP) and 15 with severe AP (S-AP) were included in our study. Peripheral blood lymphocytes were examined at d 1-3, 5, 10 and 30 by means of flow cytometry.

RESULTS: A significant depletion of circulating lymphocytes was found in AP. In the early AP, the magnitude of depletion was similar for T- and B- lymphocytes. In the late course of S-AP, B-lymphocytes were much more depleted than T-lymphocytes. At d 10, strong shift in the CD7+/CD19+ ratio implicating predominance of T-over B-lymphocytes in S-AP was found. Among T-lymphocytes, the significant depletion of the CD4+ population was observed in M-AP and S-AP, while CD8+ cells were in the normal range. Lymphocytes were found to strongly express activation markers: CD69, CD25, CD28, CD38 and CD122. Serum interleukin-2 (IL-2), IL-4, IL-5, IL-10, interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) levels were significantly increased in both forms of AP. The magnitude of elevation of cytokines known to be produced by Th2 was much higher than cytokines produced by Th1 cells.

CONCLUSION: AP in humans is characterized by significant reduction of peripheral blood T- and B-lymphocytes.

INTRODUCTION

Excessive leukocyte activation with cytokinemia represents one of the most important mechanisms of increased mortality in early acute pancreatitis (AP). It has been hypothesized that fatal pancreatitis is a consequence of excessive leukocytic phagocyte stimulation provoked by severe trauma or persistent injury due to an agent noxious to the pancreas^[1]. Lymphocytes and inflammatory mediators released by these cells are one of the most potent regulators of leukocytic phagocytes. It has been suggested that T- and B- lymphocytes activation is a key factor in the modulation of the inflammatory reaction in different diseases, including AP^[2]. The role of different inflammatory mediators in AP has already been extensively studied, while the role of lymphocyte activation and its relation to disease severity in humans are still poorly understood^[3-6]. Few studies have demonstrated the reduction of total peripheral lymphocytes as well as CD4+, CD8+, CD3+DR- and CD3-DR+ lymphocyte subsets in early severe AP^[7-10]. In the most recent study, T cell activation has been reported in mild acute pancreatitis^[11]. Another study showed that treatment with high-dose vitamin C partially restored depleted peripheral blood CD4+ cells and increased the ratio of CD4+/CD8+ cells^[12]. Under the normal or pathologic conditions, CD4+ T helper (Th) lymphocytes polarize into two major subsets: Th1 and Th2. Both environmental and genetic factors act in concert to determine the Th1 and Th2 polarization. The collective data available so far are not sufficient to determine the role of specific Th cells subsets in acute pancreatitis in humans.

In this study, we, therefore, aimed to provide broad and complex evaluation of peripheral blood lymphocyte subsets in patients with AP.

MATERIALS AND METHODS

Clinical evaluation

Thirty five patients (16 women and 19 men; age 32-77 years, median 56 years) with AP were prospectively included into our study. In all patients, the time between the abdominal pain onset and admission to the hospital was not longer than 48 h. The control group comprised of 15 healthy volunteers (7 women and 8 men; age 19-77 years, median 41 years) without the history of recent inflammatory disease. The diagnosis was made on the basis of a history consistent with AP and serum amylase activity > 3 times the upper limit of normal range (20-90 U/L). For all the patients, additional biochemical and imaging (ultrasound and computed tomography) tests have been done to confirm the diagnosis as well as to determine the etiology and severity of AP according to Ranson's^[13] and Balthazar's^[14] criteria supplemented by serum C-reactive protein (CRP) concentration measurements. At d 2-3, CRP concentrations higher than 150 mg/L were considered as indicative of S-AP. Using these criteria, our patients were divided into mild AP ($n = 20$) and severe AP ($n = 15$) (Table 1). Among the patients with S-AP, the following systemic complications were found: pulmonary in 8 patients, circulatory in 3, renal in 3, coagulation disorders in 1 and septic complications in 1 patient. Local complications, such as acute fluid collections in 10 patients, infected necrosis in 1 and pancreatic abscess in 1 patient, were found. By etiology, 18 patients presented biliary pancreatitis, 16 alcoholic pancreatitis and 1 idiopathic pancreatitis. In the course of hospitalization, 2 patients with severe AP died. Medical University of Białystok Ethical Committee approval had been granted for performing this study.

Treatment

In patients with S-AP, in addition to standard treatment, prophylactic therapy with antibiotic (meropenem 500 mg tid for 10-21 d) and enteral nutrition have been implemented since the d 2-3 of the disease.

Laboratory methods

EDTA-anticoagulated blood samples were taken at admission (d 1), on d 2, 3, 5, 10 and 30. Blood samples were drawn from the antecubital vein. The absolute number of leukocytes (granulocytes, lymphocytes, monocytes) was estimated with a hematological analyzer Advia 120 (Bayer). Surface lymphocyte antigens (CD) were assayed by the direct fluorescence method for whole blood, using a flow cytometer (EPICS XL, Coulter) and double staining (FITC/PE) monoclonal antibodies (Becton Dickinson). Briefly, 100 μ L samples of whole blood were incubated with 5 μ L of respective monoclonal antibody solution. After 30 min of incubation in the dark at 4°C, erythrocytes were lysed, while leukocytes were fixed, stabilized (ImmunoPrep, Coulter) and analysed by EPICX XL. The use of "gate-check" [CD45-FITC/CD14-PE (BD)] allowed the division of the leukocytes population into granulocytes, lymphocytes and monocytes. Matched labelled anti-idiotypic antibodies were used as negative controls (IgG1-FITC/IgG1-PE).

Table 1 Clinical characterization of studied patients with mild AP and severe AP

	Mild AP $n = 20$	Severe AP $n = 15$	P M-AP vs S-AP (Mann-Whitney U test)
Etiology			
Biliary	9 (45.0%)	9 (60.0%)	
Alcohol	10 (50.0%)	6 (40.0%)	
Idiopathic	1 (5.0%)	0 (0%)	
Median ranson score (range)	1 (0-2)	4 (2-8)	$P < 0.01$
Balthazar score			
A	6 (30.0%)	0	
B	14 (70.0%)	0	
C	0	2 (13.3%)	
D	0	5 (33.3%)	
E	0	8 (53.3%)	
CRP (mg/L) median (range)			
D 1	22.9 (5.0-139.0)	216.0 (5.0-466.1)	$P < 0.01$
D 2	50.5 (5.2-135.4)	186.2 (43.1-342.0)	$P < 0.01$

Populations and subpopulations of lymphocytes

Populations and subpopulations of lymphocytes were evaluated using the following differentiation antigens: CD19-/CD7+ (T-lymphocytes), CD3+/CD4+ (Th-lymphocytes), CD3+/CD8+ (cytotoxic T-lymphocytes) and CD19+/CD7- (B-lymphocytes).

Activation markers of T-lymphocytes

The following CD antigens were examined: CD3/CD69, CD7/CD122 and CD8/CD38, CD8/CD28.

Activation markers of B-lymphocytes

CD19/CD122 expression was determined in B-lymphocytes.

Determination of apoptosis

Cell preparation: Mononuclear cells were isolated by density gradient concentration on Histopaque 1077 (Sigma). Highly purified mononuclear cells (about 98%, by Advia 120) were obtained. The cells were subsequently suspended in tubes with phosphate-buffered saline.

Determination of spontaneous apoptosis: Apoptosis was determined using Annexin V-Fluos and propidium iodide double staining. Apoptosis is accompanied by a loss of membrane phospholipid asymmetry, resulting in the exposure of phosphatidylserine at the surface of the cell. Quantitative measurement of phosphatidylserine exposure was possible using the binding of fluorescein isothiocyanate-labelled annexin V to phosphatidylserine. For the annexin V assay, cells were incubated for 1 h at 37°C in 2 mL of buffer containing FITC-labelled annexin V and propidium iodide (PI) and line specific markers (CD3, CD19, CD33, CD14, PE). Flow cytometric analysis was performed by Coulter EPICS XL.

Determination of stimulated apoptosis

Determination of stimulated apoptosis was carried out as previously described^[15]. Isolated lymphocytes were incubated in six-well plates (Falcon, Becton Dickinson)

with RPMI 1640 medium (GIBCO) without fetal bovine serum at 37°C in a humidified atmosphere containing 50 mL/L CO₂. After 24 h of incubation, cells were stained with a dye mixture (10 µmol/L acridine orange and 10 µmol/L ethidium bromide; Sigma) prepared in phosphate-buffered saline (PBS). Two-hundred cells per sample were examined by fluorescence microscopy, and the cells were characterized according to the following criteria: (1) native cells-fine reticular pattern of green stain chromatin; (2) necrotic cells-bright orange stain chromatin; and (3) apoptotic cells-green stain chromatin which was highly condensed and uniformly stained by acridine orange.

Determination of cytokines produced by Th1/Th2 subsets

The BD Human Th1/Th2 Cytokine CBA kit was used to measure IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ levels in serum samples. The samples diluted with the appropriate volume of Assay Diluent were transferred into the assay tubes containing capture beads (covered with respective cytokine antibody) and PE detection reagent. The standard curve for each cytokine was defined for concentrations from 20-5000 ng/L. The assay was performed using a FACSCalibur (BD) flow cytometer.

Statistical analysis

Statistical analysis was performed using the non-parametric Mann-Whitney's *U* test. *P* values less than 0.05 were considered statistically significant. Results were expressed as mean ± SD.

RESULTS

At d 1, significant decrease of total lymphocyte number to 63% and 39% of the healthy control was found in patients with M-AP and S-AP, respectively (Figure 1A). In the course of the disease, lymphocyte number gradually increased, reaching 79% and 97% of the control in M-AP and S-AP at d 10, respectively. At d 30, lymphocyte number fell again to 65% and 42% of the control, respectively, in M-AP and S-AP. Analysis of CD7 and CD19 molecule expression showed that T-lymphocyte (CD7+) population was especially reduced in the early course of S-AP (Figure 1B). Thereafter, it was constantly increasing, returned to normal at d 10 and decreased again to 46% of the control at d 30. In M-AP, depletion of T-lymphocyte population was less evident (Figure 1B). Some reduction of B-lymphocyte (CD19+) number was found in the early S-AP, while marked depletion to 36% and 34% of the control was noticed at d 10 and 30, respectively (Figure 1C). In M-AP, the decrease of B-lymphocyte number was not so persistent.

Two major subsets of T-lymphocytes are represented by CD4+ and CD8+ cells, also known as T4-helper cells and T8-cells or cytotoxic T-lymphocytes (CTLs), respectively. At d 1, the number of circulating CD4+ cells significantly dropped in both M-AP and S-AP to 68% and 45% of the control, respectively (Figure 1D). In M-AP, at d 5 and 10, it returned to the range of normal level, while in S-AP it remained significantly depleted until d 30. The number of circulating CD8+ cells remained in the range

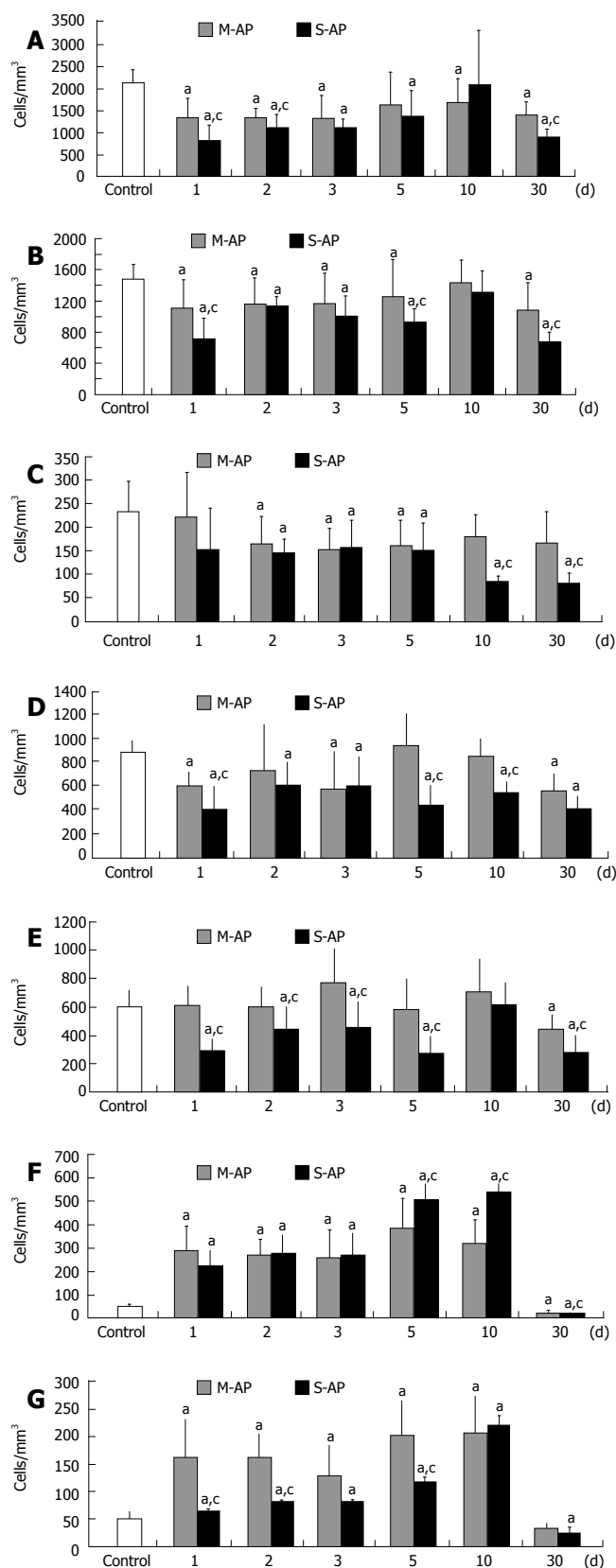


Figure 1 Peripheral blood lymphocytes in patients with AP. **A:** Total count; **B:** CD7+; **C:** CD19+; **D:** CD4+; **E:** CD8+; **F:** CD69+; **G:** CD25+. Results are expressed as mean ± SD. **P* < 0.05 vs control; °*P* < 0.05 vs mild AP at the same day of observation.

of the normal level in the early M-AP, including the d 10 (Figure 1E). At d 30, it decreased to 73% of the control.

Table 2 Peripheral blood lymphocyte CD7+/CD19+ and CD4+/CD8+ ratio in patients with AP

		Days						
		Control	1	2	3	5	10	30
CD7+/CD19+	M-AP	6.4 ± 1.9	4.9 ± 1.2 ^a	7.0 ± 2.3	7.6 ± 2.1	7.8 ± 2.1	7.9 ± 1.9	6.5 ± 1.7
	S-AP		4.6 ± 1.2 ^a	8.0 ± 2.1 ^a	6.3 ± 1.9	6.1 ± 1.8	15.8 ± 4.7 ^{ac}	8.7 ± 2.0 ^{ac}
CD4+/CD8+	M-AP	1.5 ± 0.6	1.0 ± 0.4 ^a	1.2 ± 0.5	0.7 ± 0.6 ^a	1.6 ± 0.5	1.2 ± 0.5	1.2 ± 0.5
	S-AP		1.4 ± 0.5 ^c	1.4 ± 0.6	1.3 ± 0.6 ^c	1.6 ± 0.6	0.9 ± 0.6 ^a	1.5 ± 0.5

^a*P* < 0.05 vs control; ^c*P* < 0.05 vs mild AP at the same day of observation.

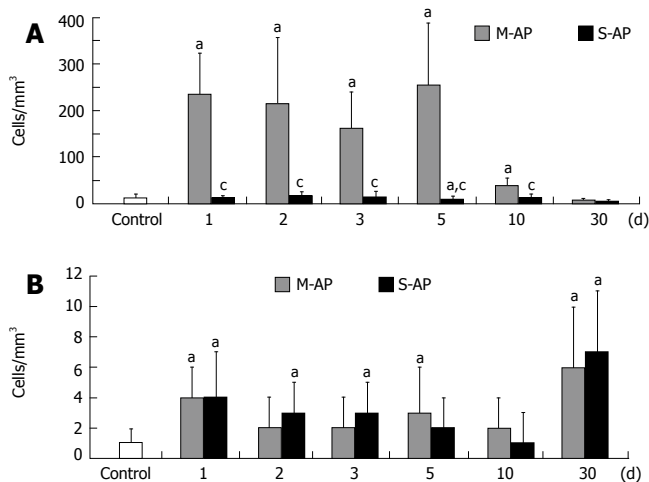


Figure 2 Peripheral blood T-lymphocytes (CD7+) (A) and B-lymphocytes (CD19+) (B) expressing CD122 in patients with AP. Results are expressed as mean ± SD. ^a*P* < 0.05 vs control; ^c*P* < 0.05 vs mild AP at the same day of observation.

At d 1 of S-AP, CD8+ cells were markedly depleted to 47% of the control and remained below normal level until d 5. CD8+ returned to normal level at d 10, and depleted again to 47% of the control at d 30.

In patients with both M-AP and S-AP, some fluctuations of lymphocyte T/B (CD7+/CD19+) ratio were observed (Table 2). At d 10, the ratio was markedly increased because the T-lymphocyte number was close to normal and the significantly diminished B-lymphocyte level. In M-AP and S-AP, the lowest CD4+/CD8+ cells ratio was found at d 3 and 10, respectively (Table 2).

Peripheral blood lymphocytes in both M-AP and S-AP patients showed a dramatic increase of intracellular signaling manifested by CD69 expression. At d 1, it was elevated 6.0-fold and 4.6-fold in M-AP and S-AP, respectively (Figure 1F). At d 10, CD69 expression in peripheral blood lymphocytes reached a maximum (11.2-fold over the control level) in S-AP and then dramatically declined to 52% and 40% of the healthy control in M-AP and S-AP, respectively.

CD25/IL-2R α expression, known as the lymphocyte proliferation marker, was elevated especially in M-AP (Figure 1G). At d 1 and 10, it increased to 3.3-fold and 4.2-fold over the control, respectively. In S-AP, a marked elevation of CD25/IL-2R α expressing lymphocytes (4.5-fold over the control) was found at the d 10. At d 30, the number of the lymphocyte subpopulation significantly decreased to 63% in M-AP and to 47% in S-AP. The

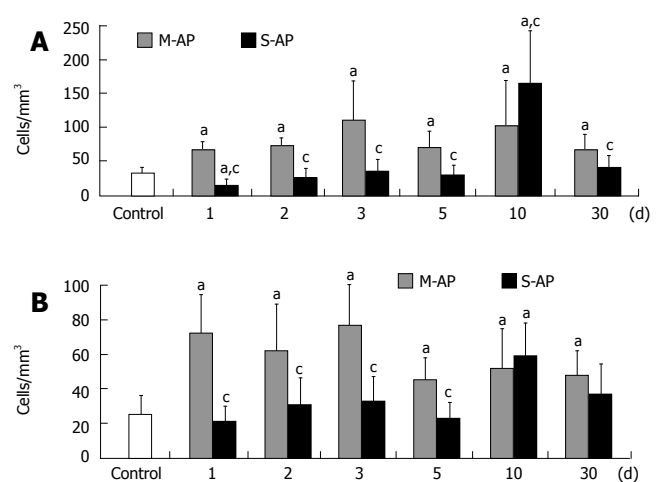


Figure 3 Peripheral blood CD8+ cells expressing CD38 (A) and CD28 (B) in patients with AP. Results are expressed as mean ± SD. ^a*P* < 0.05 vs control; ^c*P* < 0.05 vs mild AP at the same day of observation.

number of peripheral blood T-lymphocytes expressing CD122 sharply increased in patients with M-AP (Figure 2A). At d 1, it increased almost 20-fold compared to the control, and remained highly elevated until d 5. At the d 10, the number of lymphocytes significantly diminished and at d 30, reached 50% of the control level. In the course of S-AP, the number of T-lymphocytes expressing CD122 did not significantly change. However, at d 30, it dropped to 33% of the normal level.

The number of peripheral blood B-lymphocytes with the same marker of activation was significantly elevated in both M-AP and S-AP patients (Figure 2B). Interestingly, concerning CD122 expression, the highest level of B-lymphocyte activation, 6- and 7-fold were found in M-AP and S-AP at the d 30, respectively. Figure 3 shows the pattern of activation of T-lymphocyte CD8+ subset. In M-AP, the number of cells expressing CD38 increased to 20.6% of the control level at d 1, reached the maximum level of 33.9% at d 3 and remained elevated at 20.3% at d 30 (Figure 3A). In S-AP, the opposite trend was noted with significant diminishing of CD8+CD38+ lymphocytes to 45% of the control at d 1. It returned to normal level at d 3, while a strong temporary elevation to 49.7% of the control at d 10 was noted.

Concerning CD28 expression, CD8+ T-lymphocyte activation was the strongest observed in M-AP patients (Figure 3B). At d 1, it reached 28.8% of the control, remained at the similar level until d 3 and then slightly decreased to 19.2% of the control at d 30. In S-AP, it oscillated around normal

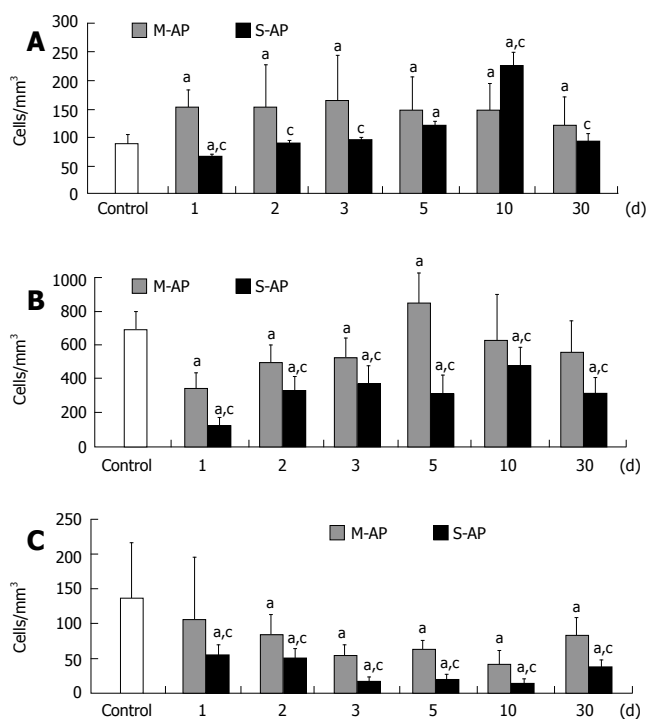


Figure 4 Markers of lymphocyte apoptosis in patients with AP. Peripheral blood lymphocytes expressing CD95 (A), CD3+ lymphocytes expressing Annexin V (B) and CD19+ lymphocytes expressing Annexin V (C). Results are expressed as mean \pm SD. ^a $P < 0.05$ vs control; ^c $P < 0.05$ vs mild AP at the same day of observation.

level until d 5, increased to 23.6% of the control at d 10, and remained slightly elevated at d 30.

Expression of CD95/Fas-R/Apo-1 receptor protein, which plays an important role in apoptosis initiation, was enhanced in early M-AP and reached a maximum (18.8% of the control) at d 3 (Figure 4A). It remained slightly elevated throughout the course of the disease. In S-AP, the number of lymphocytes expressing CD95/Fas-R/Apo-1 slightly decreased to 7.6% of the control at the d 1 and then constantly raised up to 26.8% at d 10. Thereafter, it returned to the control level at the d 30.

At d 1, the number of peripheral blood T-lymphocytes that stained for annexin V (AnnV+CD3+) significantly decreased in M-AP and S-AP to 49% and 17% of the normal level, respectively (Figure 4B). Thereafter, it gradually increased, and in M-AP slightly exceeded normal level (12.2% of control) at d 5. In S-AP, the number of AnnV+CD3+ lymphocytes remained diminished during the course of whole observation. The number of peripheral blood B-lymphocytes that stained for annexin V (AnnV+CD19+) started to decrease at d 1 in both forms of AP, and at d 10, reached the minimum value, i.e. 30% and 10% of the normal level in M-AP and S-AP, respectively (Figure 4C). Table 3 shows the viability of lymphocytes isolated from peripheral blood of patients with pancreatitis. In both forms of pancreatitis, in non-stimulated cells, the rates of apoptosis and necrosis were at similar levels as in the control. Stimulated cells from the healthy control died predominantly by necrosis. However, in stimulated cells of patients with pancreatitis, lymphocytes died predominantly by apoptosis, without

Table 3 Apoptosis and necrosis rate in lymphocytes isolated from patients with AP

	Cultured with serum (% of total)			Cultured without serum (% of total)		
	Apoptosis	Living	Necrosis	Apoptosis	Living	Necrosis
Control	2 \pm 1	96 \pm 2	1 \pm 1	23 \pm 2	10 \pm 3	68 \pm 3
M-AP	3 \pm 1	97 \pm 1	1 \pm 1	50 \pm 6 ^a	17 \pm 6	33 \pm 4 ^a
S-AP	3 \pm 1	96 \pm 2	2 \pm 2	50 \pm 6 ^a	15 \pm 4	36 \pm 5 ^a

^a $P < 0.05$ vs control.

Table 4 Serum concentration of cytokines produced by Th1 lymphocyte subpopulation in patients with AP

		Days					
		Control	1	2	3	5	10
IL-2	6.2 ± 5.4	M-AP	20 ± 6 ^a	18 ± 8 ^a	18 ± 7 ^a	12 ± 7 ^a	8 ± 4 ^a
(ng/L)		S-AP	21 ± 10 ^a	24 ± 11 ^{ac}	25 ± 11 ^{ac}	13 ± 9 ^a	9 ± 5 ^a
IFN-γ	6.0 ± 5.7	M-AP	45 ± 24 ^a	44 ± 25 ^a	41 ± 14 ^a	28 ± 11 ^a	17 ± 5 ^a
(ng/L)		S-AP	258 ± 24 ^{ac}	49 ± 23 ^a	40 ± 19 ^a	25 ± 10 ^a	16 ± 6 ^a
TNF-α	1.8 ± 2.4	M-AP	2.9 ± 1.2 ^a	2.9 ± 1.6 ^a	1.5 ± 0.9	0.9 ± 0.7 ^a	1.0 ± 0.9 ^a
(ng/L)		S-AP	3.2 ± 1.4 ^a	3.2 ± 1.5 ^a	1.0 ± 1.0 ^{ac}	1.0 ± 0.7 ^a	1.1 ± 0.9 ^a

^a $P < 0.05$ vs control; ^c $P < 0.05$ vs mild AP at the same day of observation.

Table 5 Serum concentration of cytokines produced by Th2 lymphocyte subpopulation in patients with AP

		Days					
		Control	1	2	3	5	10
IL-4	6.0 ± 5.8	M-AP	164 ± 57 ^a	133 ± 60 ^a	79 ± 34 ^a	82 ± 43 ^a	60 ± 33 ^a
(ng/L)		S-AP	198 ± 74 ^{ac}	175 ± 68 ^{ac}	92 ± 42 ^{ac}	94 ± 45 ^{ac}	72 ± 35 ^{ac}
IL-5	5.2 ± 4.9	M-AP	19 ± 9 ^a	29 ± 9 ^a	17 ± 7 ^a	52 ± 23 ^{ac}	4 ± 3
(ng/L)		S-AP	23 ± 7 ^a	40 ± 12 ^{ac}	18 ± 7 ^a	39 ± 19 ^{ac}	3 ± 3
IL-10	1.4 ± 1.6	M-AP	40 ± 27 ^a	50 ± 33 ^a	49 ± 27 ^a	32 ± 19 ^a	39 ± 23 ^a
(ng/L)		S-AP	52 ± 36 ^{ac}	62 ± 36 ^{ac}	52 ± 36 ^a	50 ± 36 ^{ac}	47 ± 31 ^{ac}

^a $P < 0.05$ vs control; ^c $P < 0.05$ vs Mild AP at the same day of observation.

showing any significant difference between M-AP and S-AP.

We measured serum concentrations of cytokines produced by Th1 and Th2 lymphocytes in the first 10 days of the disease (Tables 4, 5). IL-2, IFN- γ and TNF- α represent the pattern characteristic for Th1, while IL-4, IL-5 and IL-10 are produced by Th2. IL-2 and IFN- γ levels were much higher in S-AP than in M-AP, while TNF- α was slightly elevated in both forms of pancreatitis (Table 4). At d 1, we observed a huge increase by 27-fold and 33-fold in the IL-4 level in M-AP and S-AP, respectively (Table 5). It remained highly elevated in the whole early course of pancreatitis. IL-5 was significantly increased in both forms of pancreatitis during the first 5 d of observation. However, it returned to normal level at d 10. A remarkable increase of IL-10 was noted in both forms of pancreatitis. The peak, 36-fold and 44-fold elevation, was observed in M-AP and S-AP at d 2, respectively (Table 5).

DISCUSSION

Alteration of the immune system is one of the major mechanisms responsible for early and late mortality in severe AP. Excessive inflammatory reaction, known as systemic inflammatory response syndrome (SIRS), is considered as the leading cause of death in early AP^[4-6]. The role of lymphocytes in this phenomenon has been partly studied and the knowledge of the mechanisms in humans is still incomplete^[7-11,16]. Our study provides, probably for the first time, a broad and complex analysis of peripheral blood lymphocyte subsets with reference to different time points and different severity forms of acute pancreatitis. In agreement with the previous studies^[7-11,16], we also found a significant depletion of circulating lymphocytes which was much more profound in the severe form of pancreatitis. In the early period of 1-5 d of the disease, the magnitude of peripheral depletion was similar for T- (CD7+) and B- (CD19+) lymphocytes. However, in the second week and at d 30 of S-AP, B-lymphocytes were found to be particularly depleted. Concerning the relations between these subsets of lymphocytes, the middle of the second week (d 10) was critical because of the strong predominance of T- over B-lymphocytes in S-AP. Depletion of the CD19+ lymphocyte subset in early acute pancreatitis has also been recently reported^[16]. Among T-lymphocytes, at d 1 and 3 of M-AP as well as at d 10 of S-AP, we observed significant depletion of the CD4+ population, while CD8+ cells were in the normal range. It created a temporary imbalance in the cell ratio with the apparent prevalence of CD8+ over the CD4+ cells. CD8+ cells are known as cytotoxic T-lymphocytes (CTL) and some of them differentiate into T8-suppressor cells. Significant depletion of peripheral blood CD4+ and CD8+ subpopulations of T-lymphocytes in the course of acute pancreatitis has previously been reported^[7-9,12,16]. However, observations made by the others were restricted mostly to one or up to three different time periods of pancreatitis.

The spectrum of cytokines known to modulate lymphocyte differentiation and function (IL-2, IFN- γ , TNF- α , IL-4, IL-5 and IL-10), evaluated in the early course of pancreatitis, showed all of them significantly increased in both forms of AP, however, the highest values were observed in S-AP. Previous studies showed the elevated IL-2, TNF- α and IL-10 levels in human AP^[11,12,17,18]. To our knowledge, no data are available in literature on serum levels of IFN- γ , IL-4 and IL-5 in human AP. In our study, the magnitude of elevation of cytokines known to be produced by Th2 cells, namely IL-4, IL-5 and IL-10, was much higher than cytokines produced by Th1 cells, represented by IL-2 and IFN- γ . This finding suggests that in the course of AP, Th1 subpopulation of CD4+ cells is suppressed more strongly than Th2. Early IL-4 expression during an immune response is critical for determining the development of Th2 cells^[19]. Cytokines produced by Th2 cells enable activated B-lymphocytes to proliferate, stimulate activated B-lymphocytes to synthesize and secrete antibodies, promote the differentiation of B-lymphocytes into antibody-secreting plasma cells, and enable antibody-producing cells to switch the class of antibodies being

produced^[20]. In our study, B-lymphocyte activation was the strongest at d 30, while T-lymphocyte activation remained normal or even below the control level. This pattern of lymphocyte activation might be the consequence of shift from Th1 to Th2 in patients with AP. Th1 and Th2 cells represent polarized forms of the CD4+ T cell-mediated immune response. Th1 cells produce IL-2, IFN- γ and TNF- α which cooperate with B-cells for the production of antibodies, activate phagocytic cells and CD8+ T-cells, thus promoting cell-mediated immunity and cytotoxic T-cell responses^[21]. In contrast, cytokines released by Th2 cells (IL-4, IL-5, IL-9 and IL-13) induce B-cells to produce high amounts of IgG4 and IgE in humans, promote the differentiation and growth of mast cells and eosinophils, and inhibit several phagocytic functions^[21]. Of note, while IL-4 inhibits the development of Th1 cells, IFN- γ inhibits the development of Th2 cells^[21]. Treg cells are a highly heterogeneous family, which includes type 3 Th (Th3) cells, T regulatory 1 (Tr1) cells, and CD4+ CD25+ T cells. Interestingly, Tr1 cells are mainly able to produce IL-10. Therefore, the huge elevation of serum IL-10 level found in our study may suggest that Tr1 cells are especially active in the course of AP.

In our study, significant activation of lymphocytes was observed, which was shown by strong expression of CD69, CD25, CD28, CD38 and CD122 on T-lymphocytes as well as CD122 on B-lymphocytes. The surface receptors CD69 and the IL-2 receptor (CD25) are early markers of activation. Increased expression of CD69 as well as CD25 on CD3+, CD4+ and CD8+ cells has recently been reported in mild AP^[11]. It has also been reported that the number of B-lymphocytes (CD19+) expressing CD69+ was significantly lower in patients with severe pancreatitis than in patients with mild pancreatitis^[16]. Consequently, the conclusion has been made that patients with severe pancreatitis show impaired early activation of peripheral CD19+ cells. Interestingly, in our study, evaluation of CD19+ cells expressing CD122, which is another marker of lymphocyte activation, showed significant activation of the B-lymphocyte subset bearing this marker. These observations may suggest that the CD19+ cell subset is heterogeneous and the subsets of these cells react in a different manner in the course of pancreatitis.

Marked activation of different subsets of lymphocytes may explain why we have observed strong elevation of different cytokines in peripheral blood despite a significant decrease in the number of circulating lymphocytes. The question remains, however, what was the reason for significant depletion of peripheral blood lymphocytes in pancreatitis. One of the possible reasons is strong migration of activated lymphocytes to the site of inflammation, including the pancreas and other tissues like lungs or kidneys, as a part of SIRS^[22]. Another possible explanation is an excessive elimination of lymphocytes by apoptosis. Expression of CD95, known as Apo-1, Fas or death receptor on lymphocytes was increased in early M-AP and in S-AP at d 10. Surprisingly, the expression of annexin V, which is a marker of ongoing apoptosis, was decreased in early and late S-AP on both T- and B-lymphocytes. In the late period of M-AP, annexin V expression on T-lymphocytes was in the normal range,

while it was decreased on B-lymphocytes. This finding is in agreement with our observation of increased activation of B-lymphocytes in the late course of pancreatitis. It is known that stimulation by factors inducing cell proliferation may inhibit apoptosis^[23]. This phenomenon is the basis of the method evaluating cell susceptibility to apoptosis^[15]. Using this *in vitro* method, we found that lymphocytes from patients with pancreatitis were primed to apoptosis. Similar results of *in vitro* incubation of lymphocytes from patients with S-AP have previously been reported^[10]. Taken together, peripheral blood lymphocyte depletion in acute pancreatitis may result from both excessive apoptosis and migration to the site of inflammation.

Tissue damage caused not only by severe AP but also by traumatic incidents, like severe burns, accident trauma, major surgical interventions or sepsis, induces commensurate with the severity of damage (damage load), genetic factors (gene polymorphism), the general condition of the host and the type of antigens (antigenic load), both local and systemic release of pro-inflammatory cytokines and phospholipids^[24-26]. Polymorphonuclear leukocytes, monocytes, tissue macrophages, lymphocytes, natural killer cells, and parenchymal cells are involved in a complex network of the host defense response. An overwhelming pro-inflammatory response (hyper-inflammation) leads to the clinical manifestations of SIRS and finally to host defense failure expressed by multiple organ dysfunction syndrome (MODS) or multiple organ failure (MOF). The up-regulation of pro-inflammatory factors, such as TNF, IL-1 and IL-6, observed during the SIRS phase can be followed by a second response that involves down-regulation of IFN- γ and increase in anti-inflammatory cytokines, such as IL-10 and transforming growth factor- β . This counter-regulatory phenomenon is called the compensatory anti-inflammatory response syndrome (CARS)^[24]. Aside from cytokine profiles, critical injury and sepsis are also correlated with dysfunction of many immune cells. After severe injury, CD4+ T-cell differentiation into Th phenotypes is altered and there is an early expression of Th1 cytokines (IL-12, IFN- γ), followed 24 to 72 h later by a predominance of the Th2 cytokine (IL-4) and depression in the production of IL-2 and IFN- γ ^[24]. It has been found that severe AP, burns, accident trauma, major surgical interventions or sepsis are associated with a significant decrease in total systemic lymphocyte counts, including both CD4+ and CD8+ cells. The development of immunosuppression in subjects suffering from traumatic events is often associated with elevation of IL-10 and the shift of the Th1/Th2 balance towards a Th2 response^[24,26]. The majority of data about lymphopenia in trauma-related cellular immune defects refer to T-cells with scant information about B-cells. Lung and/or kidney failure may take place in the course of specific diseases affecting these organs or may be a part of MOF or MODS resulting from tissue damaging events, including S-AP. It has been found that peripheral blood B-cells, but not CD4+ and CD8+, were significantly lower in uremic patients. This phenomenon may be partially attributed to an increased susceptibility to apoptosis associated with a decreased expression of

Bcl-2^[27]. On the contrary, T lymphopenia with normal counts of peripheral blood B-cells has recently been reported in end-stage renal disease^[28,29]. *Ex vivo* evaluation of T-cells showed an increased number of annexin V and CD95 (Fas)-positive T-cells, suggesting that apoptosis may be responsible for excessive elimination of this lymphocyte subpopulation^[28]. A progressive decrease in renal function associated with activation and selective loss of naïve CD4+ and CD8+ T-cells as well as CD4+ central memory cells has recently been reported. These changes may contribute to the clinical phenomena of the uremia-associated immune defect in patients with chronic renal disease^[29]. Acute respiratory distress syndrome (ARDS), a sudden, life-threatening lung failure, can complicate the course of severe AP and other diseases with critical tissue damage. Severe lymphopenia has recently been reported in patients with severe acute respiratory syndrome (SARS) who developed ARDS^[30]. ARDS occurring in renal transplantation patients with pneumonia is accompanied by a significant decrease in blood CD4+ and CD8+ T cells. In these patients, the recovery of the disease met the recovery course of their immune system^[31]. In conclusion, the available data indicate that lymphopenia caused by the decrease of different lymphocyte subpopulations is characterized not only for severe AP but also for other diseases with severe tissue damage. However, with the exception of our study, the available data provide mostly the information about a single or just a few representatives of lymphocyte subpopulations in a specific disease. Further studies involving broad spectra of immune processes regulation are necessary in order to better understand the complex phenomenon of immunity disorders in diseases with critical tissue damage.

In summary and conclusion, in patients with acute pancreatitis, we found a significant depletion of circulating lymphocytes which was much more profound in the severe form of the disease. In the early period of pancreatitis, the magnitude of peripheral depletion was similar for T- and B- lymphocytes. However, in the late course of S-AP, B-lymphocytes were particularly depleted. The second week of the disease seems to be critical for the cellular immunity function, because of the strong shift in the CD7+/CD19+ ratio implicating predominance of T- over the B-lymphocytes in S-AP. Among T-lymphocytes, the significant depletion of CD4+ population was observed in M-AP and S-AP, while CD8+ cells were in the normal range. It created a temporary imbalance in the cell ratio with the apparent prevalence of CD8+ over the CD4+ cells. Serum IL-2, IFN- γ , TNF- α , IL-4, IL-5 and IL-10 levels were significantly increased in both forms of AP, with the highest values found in S-AP. The magnitude of elevation of cytokines known to be produced by Th2 cells was much higher than cytokines produced by Th1 cells. This finding suggests that in the course of AP, Th1 subpopulation of CD4+ cells is suppressed more strongly than Th2. The pattern of lymphocyte activation we found in AP patients may be the consequence of shift from Th1 to Th2. Strong elevation of serum level of different cytokines, despite a significant decrease in the number of circulating lymphocytes, may be explained by the significant activation of T- as well as B-lymphocytes. Peripheral blood

lymphocyte depletion in acute pancreatitis may result from both excessive apoptosis and migration to the site of inflammation. The data obtained in this study systematize our current knowledge on different lymphocyte subsets in acute pancreatitis and show avenues for future research on cellular immunity in this disease.

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RAPID COMMUNICATION

Association of rare *SPINK1* gene mutation with another base substitution in chronic pancreatitis patients

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the same gene copy. Most probably the 5'UTR-215 G > A represents a rare polymorphism and not a mutation as previously concluded. Haplotype analysis suggests a common origin of the IVS3 + 2 T > C mutation in these patients.

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Key words: Chronic pancreatitis; Serine protease inhibitor Kazal type 1; Gene mutations

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Abstract

AIM: To verify and expand the known spectrum of serine protease inhibitor Kazal type 1 (*SPINK1*) gene mutations in chronic pancreatitis.

METHODS: DNA extracted from 172 chronic pancreatitis patients was assayed for *SPINK1* gene mutations by PCR and DNA sequencing. A control cohort of 90 unrelated healthy individuals was analysed by the same methods for presence of common populational polymorphisms, and frequency of five-loci haplotypes was calculated. Linkages of gene aberrations in single *SPINK1* gene copies were analysed by long-distance PCR followed by allele-specific PCR and DNA sequencing.

RESULTS: The most frequent *SPINK1* gene mutation N34S was found at a frequency of 6%. Furthermore, we detected the heterozygous intervening sequence (IVS) 3 + 2 T > C mutated gene in 2 German patients and 1 Macedonian chronic pancreatitis patient. In all three *SPINK1* gene copies an additional rare base substitution was found: 5'untranslated region (UTR)-215 G > A. Polymorphism analysis revealed that all three affected genes carried the same five-loci haplotype. DNA sequencing of another chronic pancreatitis-related gene *PRSS1* (cationic trypsinogen) did not reveal any mutations in these 3 patients.

CONCLUSION: We found in 3 (2%) of 172 chronic pancreatitis patients an IVS3 + 2 T > C *SPINK1* gene mutation and a base substitution 5'UTR-215 G > A in

INTRODUCTION

In approximately one-third of all patients with chronic pancreatitis, no etiological factor can be found, and these patients are classified as having idiopathic disease. Recent genetic discoveries have added much to our understanding of 'chronic pancreatitis' [1]. The identification of a clear 'gain-of-function' mutation, R122H, in a cationic trypsinogen gene (*PRSS1*) that causes hereditary pancreatitis has been of high significance [2]. Loss of trypsin inhibitor function may cause pancreatitis. Several studies have demonstrated mutations in the serine protease inhibitor Kazal type 1 (*SPINK1*) gene (MIM#167790), also known as pancreatic secretory trypsin inhibitor gene (*PSTI*). This gene is approximately 7.5 kb long, consists of 4 exons and is located on chromosome 5 [3]. It encodes a polypeptide of 79 amino acids that is processed into a mature active protein consisting of 56 amino acids. Due to its molar ratio of 1:5 to trypsin, it inhibits about 20% of its activity. *SPINK1* gene variations are clearly associated with chronic pancreatitis [4,5]. They are probably a disease modifying factor, possibly by lowering the threshold for development of chronic pancreatitis caused by other genetic or environmental factors [4,6,7].

The most frequent mutation of human *SPINK1* gene is a heterozygous or homozygous missense mutation of codon 34 in exon 3 (c.101 A > G), and its incidence is about 80% in familial and idiopathic chronic pancreatitis [4,5].

This mutation leads to an A to G transition resulting in substitution of asparagine by serine (N34S). The high prevalence of this mutation in chronic pancreatitis led to the hypothesis that the N34S mutation could reduce the antiproteolytic activity of *SPINK1* subjects^[7,8].

To date, many variants of the *SPINK1* gene have been reported, however other mutations than N34S are rare (for an up-to-date list of pancreatitis-associated *SPINK1* mutations visit the website^[9]). Concerning other *SPINK1* gene sequence abnormalities, only two of them can probably be recognised as true mutations. Mutation c.2 T > C (M1T) destroys the translation initiation codon^[5]. The base substitution intervening sequence (IVS)3 + 2 T > C (also named c.194 + 2 T > C) destroys the splicing of exons 3 and 4, and was described in approximately 10 cases^[9]. A splicing mutation IVS2 + 1 G > A (c.87 + 1 G > A) and the frameshift mutations c.27delC and 98insA, both detected in single families, may be fatal for protein synthesis because of preliminary termination of the translation^[10]. The base substitution 5' untranslated region (UTR)-215 G > A in the untranslated 5'-region of *SPINK1* has been reported in two Japanese chronic pancreatitis families up to now, but in no healthy individuals^[11].

The aim of this study was to verify and extend the known spectrum of *SPINK1* gene mutations. We found 3 chronic pancreatitis patients carrying an IVS3 + 2 T > C *SPINK1* gene mutation together with a base substitution 5'UTR-215 G > A in the same gene copy. Our finding implicates that the 5'UTR-215 G > A substitution most probably represents a rare polymorphism and not a mutation as previously concluded^[11]. The same haplotypes in these genes suggest a common origin of the IVS3 + 2 T > C mutation in all 3 patients.

MATERIALS AND METHODS

Patients and controls

This study was approved by the ethics committee of the Chamber of Physicians in Hamburg, Germany. Written informed consent was obtained from all patients for use of tissue and blood samples. One hundred and seventy-two patients with chronic pancreatitis who underwent surgery in the Clinic for General, Visceral and Thoracic Surgery, University Medical Center Hamburg-Eppendorf, Germany between 1992 and 2002 were included. Pancreatic disease was confirmed by histopathological evaluation of all cases. Blood samples of 90 unrelated and healthy individuals (donors) were used as a source of control DNA and for the study of populational polymorphism.

DNA extraction and PCR

Genomic DNA from peripheral blood leukocytes and tissue specimens was extracted and purified according to the established protocols using the QIAamp blood-tissue kit (Qiagen, Hilden, Germany). All four exons of the *SPINK1* gene were amplified by polymerase chain reaction (PCR) using Taq DNA-polymerase (Perkin-Elmer, Foster City, USA) and oligonucleotide primers as previously described^[5]. PCR products were analysed by electrophoresis on a 2% agarose gel and purified by the QIAquick spin PCR-purification kit (Qiagen). Direct

DNA sequencing of the PCR products was carried out using the BigDye terminators sequencing kit (Perkin-Elmer, Foster City, USA) and nested primers on an automatic sequencer ABI 377 (Perkin-Elmer). The presence of the 5'UTR-215 G > A substitution was also tested by performing *Bgl*I restriction endonuclease digestion and gel electrophoresis analysis. *SPINK1* genes from chronic pancreatitis patients carrying the substitutions IVS3 + 2 T > C and 5'UTR-215 G > A were amplified by PCR to generate two fragments (5'-UTR-exon 3 and exon 3-3'-UTR). The long-distance PCR was performed in a 100 µL reaction mix supplemented with the TaqPlus long polymerase mixture, a high-salt buffer (Stratagene, La Jolla, USA) and the following primers: *SPINK1* pr-F 5'-TTTGAGTTCATCTTACAGGTGAG and *SPINK1* 3-R 5'-GTTTGCTTTTCTCGGGGTGAG, *SPINK1* 3-F 5'-CCAATCACAGTTATTCCCCAGAG and *SPINK1* 4-R 5'-CCAAAGTCCCCTGACCCTGG. PCR products were analysed electrophoretically on an 0.8%-1% agarose gel and purified as described above. Allele-specific PCRs were performed in a 100 µL reaction with 1 µL of long-distance PCR products as templates and Taq DNA-polymerase. The following primers were used: Spl3-T-R 5'-AAGAACTCAAGTTTGTACTCA or Spl3-C-R 5'-AAGAACTCAAGTTTGTACTCG and *SPINK1* pr-F, Pr-215G-F 5'-CATGTTTCAGGCCACCTGG or Pr-215A-F 5'-CATGTTTCAGGCCACCTGA and *SPINK1* 3-R for amplification of the 5' region of the *SPINK1* gene; Spl3-T-F 5'-GTGTTATGTTTGTGAAAATCGGT or Spl3-C-F 5'-GTGTTATGTTTGTGAAAATCGGC and *SPINK1* 4-R for amplification of the 3' region of the *SPINK1* gene. The amplification was performed by 10-15 PCR cycles using 0.5 units of Taq DNA-polymerase. The products of the allele-specific PCRs were analysed by gel electrophoresis on an 0.8%-1% agarose gel and purified as described above. For detection of the IVS3 + 2 T > C and 5'UTR-215 G > A substitutions and polymorphisms the amplified DNA was sequenced.

Haplotype analysis

The frequency of polymorphic variations in the normal population was estimated from the results obtained from DNA sequencing of the appropriate amplified DNA fragments which were extracted from leukocytes of the peripheral blood from 90 healthy unrelated individuals (180 alleles). The IVS2-352 A/G polymorphism was additionally assessed by *Msp*I restriction endonuclease digestion. The distribution of haplotypes in normal subjects for single-nucleotide polymorphic loci (SNP's) was calculated by the expectation-maximisation-algorithms (EM-algorithms) described by Weir^[12] for two-, three- and four-loci haplotypes, and according to a method proposed^[13] for two- to five-loci haplotypes. The comparison of the calculated results and probabilities of both methods led to concordant data.

RESULTS

DNA sequencing revealed mutations in *SPINK1* gene

To determine the frequency of *SPINK1* gene aberrations, DNA was isolated from consecutive surgical specimens

Table 1 Haplotype frequencies at five positions of the *SPINK1* gene from 90 individuals (180 chromosomes) as estimated by EM-algorithm

Haplotype	IVS2-352 A/G	IVS3-1643 G/C	IVS3-1568 C/A	IVS3-476 T/G	IVS3-321 C/T	n _{hpl} ¹	Haplotype frequency ²
1	A	G	C	T	C	61	0.338889
2	G	G	C	T	C	35	0.194444
3	A	G	C	T	T	4	0.022222
4	A	G	A	T	C	2	0.011111
5	G	G	C	T	T	6	0.033333
6	G	G	C	G	C	2	0.011111
7	G	G	A	T	C	7	0.038889
8	A	G	A	T	T	1	0.005556
9	A	C	A	T	C	2	0.011111
10	G	G	A	T	T	28	0.155556
11	G	G	A	G	C	1	0.005556
12	G	C	A	T	C	4	0.022222
13	G	G	A	G	T	1	0.005556
14	G	C	A	T	T	25	0.138889
15	G	C	A	G	T	1	0.005556
Total	70/110	148/32	108/72	175/5	114/66	180	1.0

¹ Estimated number of haplotypes. ² The remaining 17 possible haplotypes could not be found in any individual.

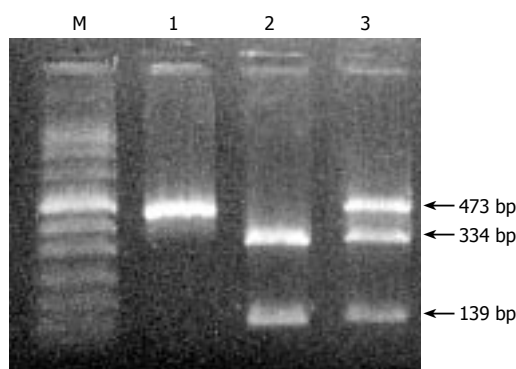


Figure 1 Detection of 5'UTR-215 G/A heterozygote in *SPINK1* gene by *Bgl*I restriction endonuclease cleavage. 1: untreated prF-1R PCR fragment; 2: normal (-215 G/G) prF-1R PCR fragment treated with *Bgl*I endonuclease; 3: heterozygote (-215 G/A) prF-1R PCR fragment treated with *Bgl*I endonuclease [M: marker (Boehringer-Mannheim marker VIII); bp: base pairs].

and blood samples of 172 patients suffering from chronic pancreatitis and analysed for *SPINK1* gene mutations. The most frequent mutation found in the *SPINK1* gene of chronic pancreatitis patients examined in our study was the N34S (A > G in position c.101), but with a rather low frequency of about 6% (10 of 172 patients).

In 3 (2%) of 152 patients a heterozygous IVS3 + 2 T > C splicing mutation was detected. DNA sequencing revealed the presence of another heterozygous base substitution in the same gene copy in the promoter region of exon 1. The detection of the 5'UTR-215 G > A substitution was also confirmed by *Bgl*I restriction endonuclease cleavage and subsequent gel electrophoresis analysis (Figure 1).

Both mutations IVS3 + 2 T > C and 5'UTR-215 G > A were present in the same gene copy

Genomic DNA from IVS3 + 2 T > C and 5'UTR-215 G > A carrying patients was amplified into two long DNA fragments (each about 4 kb) by long-distance PCR using *SPINK1* gene-specific primers. To examine the linkage

of IVS3 + 2 T > C/5'UTR-215 G > A mutations and *SPINK1* gene polymorphisms (Table 1), allele-specific PCR with a limited number of cycles was performed. Whereas the discrimination of alleles in the PCR reaction was incomplete, DNA sequencing could demonstrate the enrichment of the A peak at the position 5'-UTR-215 of the product which was synthesized with the specific primer Spl3-C-R, and, accordingly, the enrichment of the G peak of the product which was synthesized with the specific primer Spl3-T-R (Figure 2). Concordant results were found for all three patients. Based on these findings, we concluded that both single-nucleotide substitutions IVS3 + 2 T > C and 5'UTR-215 G > A were located in the same *SPINK1* gene copy of the diploid genome.

Family history of IVS3 + 2C/5'UTR-215A gene carriers was disclosed

The investigation of the relatives revealed that one patient inherited both gene aberrations of the same *SPINK1* gene copy from his mother. Interestingly however, the patient did not suffer from chronic pancreatitis but from celiac disease. These results might be a hint for a role of *SPINK1* gene mutations in celiac disease. Unfortunately, the family history of the other 2 patients was not available.

Haplotype analysis suggested a common origin of IVS3 + 2C/5'UTR-215A mutations

The haplotypes, composed of five common SNP markers, were evaluated in a population of 90 healthy individuals (blood donors) and calculated by using an EM-algorithm^[12,13]. The observed frequencies of 15 out of 32 possible haplotypes are presented in Table 1. The remaining 17 haplotypes could not be found in any individual. Long-distance PCR followed by allele-specific PCR and subsequent DNA sequencing of the IVS3 + 2C/5'UTR-215A-mutant genes showed that all three genes carried the most frequent haplotype 1. In contrast, the haplotype of the other IVS3 + 2C/5'UTR-215A-free *SPINK1* gene copy of the heterozygous gene set of all three patients

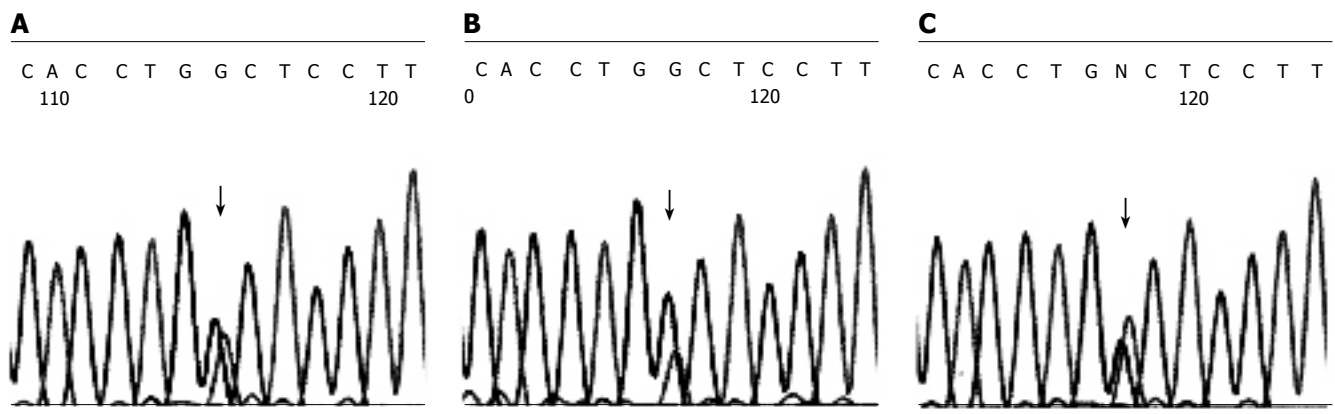


Figure 2 Linkage between 5'UTR-215 G > A and IVS 3 + 2 T > C mutations in SPINK1 gene. **A:** sequencing of -215 G/A heterozygote; **B:** sequencing of IVS 3 + 2 T (normal allele) enriched by allele-specific PCR; **C:** sequencing of IVS 3 + 2 C (mutant allele) enriched by allele-specific PCR.

appeared to be random. One of them represented the same most frequent haplotype 1, one belonged to haplotype 2 and the third to the relatively rare haplotype 6. In spite of the low number of mutant samples, these findings suggested a common origin of the IVS3 + 2C/5' UTR-215A mutation in these patients. The conclusion of a common origin is supported by the assumption that 5' UTR-215A is a rare polymorphism and not a mutation as previously proposed^[11].

No mutations of PRSS1 gene could be detected in IVS3 + 2C/5'UTR-215A carriers

DNA sequencing did not reveal any mutation in the other chronic pancreatitis-related gene *PRSS1* in these three patients.

DISCUSSION

The most frequent variation of the human *SPINK1* gene is the heterozygous or homozygous c.101 A > G (N34S) transition representing about 80% of all *SPINK1* aberrations found in familial and idiopathic chronic pancreatitis^[4,5]. In our study we detected the N34S with a frequency of 6% in chronic pancreatitis patients. In accordance with previous reports, this substitution is also the most frequent mutation of the *SPINK1* gene. Other mutations of this gene are rare, but may be of high interest concerning the role of SPINK1 in development of hereditary chronic pancreatitis. More than 10 proposed *SPINK1* gene mutations have been described until now^[9]. However, to avoid incorrect interpretation, functional analysis has to be done to investigate the influence of the suggested mutations on the activity of the *SPINK1* gene. Particularly, examination of the artificially produced N34S mutant protein demonstrated no change in its trypsin-binding ability differing from the wild-type protein^[14]. On the other hand, an inactivating effect could be postulated *a priori* for other *SPINK1* gene mutations, like IVS3 + 2 T > C, that destroys the splicing site of exons 3 and 4 and consequently leads to inactivation of the gene. We found three of these IVS3 + 2 T > C *SPINK1* mutations in two German and a Macedonian patient with chronic pancreatitis. Another base substitution, 5'UTR-215 G > A,

was also detected in all three mutant gene copies and for the first time in Caucasians. Up to now this substitution has only been detected as a homozygous base substitution in two chronic pancreatitis patients from Japan. Supported by the fact that this abnormality has never been found in healthy controls it has been considered to be an inactivating *SPINK1* gene mutation^[11]. Furthermore, 1 of the 2 Japanese patients had a family history of chronic pancreatitis. In our study, we only detected the 5'UTR-215 G > A substitution in direct linkage with the known *SPINK1* gene inactivating mutation IVS3 + 2 T > C. The probability of the occurrence of 2 true mutations in the same gene copy seems to be rather seldom. Therefore, our conclusion is that the associated base substitution 5' UTR-215 G > A in the same *SPINK1* gene copy is a rare polymorphism rather than a mutation. This assumption is supported by the fact that this base substitution is located outside the known coding and controlling sequences of the *SPINK1* gene^[11].

All 3 *SPINK1* gene copies carrying these 5'UTR-215A/IVS3 + 2C aberrations had an identical haplotype of common polymorphism, namely haplotype 1. Although only 3 cases were discovered in our study, this finding hints for a single origin of the IVS3 + 2C mutation in these 3 patients. This can also substantiate that the 5'UTR-215 G > A base substitution is rather rare.

Family data were available for one patient with 5'UTR-215A/IVS3 + 2C mutant *SPINK1* gene. This mutated gene was inherited from his mother who did not suffer from chronic pancreatitis, but from celiac disease. In some families the celiac disease was mapped close to the *SPINK1* locus on chromosome 5 (5q31)^[15,16]. Our results might be the first data suggesting a role of *SPINK1* gene mutations in celiac disease.

The data from Kume *et al.*^[17] are compatible with our findings described in the present study. They showed that the 5'UTR-215 G > A and IVS3 + 2 T > C substitutions were in complete linkage in 9 of 116 pancreatitis patients. The published frequency of 8% in this report is higher than our incidence of 2% and suggests that the linkage of both substitutions may rather play a role in Japanese patients^[17]. It would be interesting to compare the haplotypes of these mutant genes in both populations,

Mongoloids and Caucasians, to evaluate the origin of these mutations.

Taken together, we found 3 chronic pancreatitis patients carrying an IVS3 + 2 T > C *SPINK1* gene mutation and the rare base substitution 5'UTR-215 G > A in the same gene copy. Most probably, the 5'UTR-215 G > A substitution represents a rare polymorphism and not a mutation as previously concluded^[1]. Future studies with a larger patient sample and functional analysis of the *SPINK1* gene mutations are required to closer evaluate our findings and increase our understanding of their clinical manifestations.

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Autopsy study of anatomical features of the posterior gastric artery for surgical contribution

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Abstract

AIM: To investigate features of the posterior gastric artery (PGA) with respect to incidence, location and size by using autopsy subjects.

METHODS: Autopsies were performed on 72 cadavers of adults with no history of abdominal operations. The localization of the PGA, the distance between the root of the splenic artery and the origin of the PGA, and the external diameter of the PGA were examined.

RESULTS: The PGA was recognized in all patients. In 70 (97.2%) cadavers, the PGA branched from the splenic artery, and one female in this group had two PGAs. In 1 (1.4%) patient, the PGA originated from the root of the celiac trunk and in another (1.4%) patient, the PGA branched from the superior polar artery. Overall, the PGA extended for a length of 5.8-12.2 (mean, 8.4) cm from the root of the splenic artery, and the external diameter of the PGA was 1.2-3.2 (mean, 2) mm.

CONCLUSION: The anatomical features of the PGA can be readily observed and characterized by autopsy. This study has provided valuable information on the features of the PGA useful in the planning of surgical treatment.

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Key words: Posterior gastric artery; Gastric carcinoma; Autopsy

Okabayashi T, Kobayashi M, Nishimori I, Yuri K, Miki T,

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INTRODUCTION

Blood is supplied to the stomach *via* four main arteries including the right/left gastric artery, the right/left gastroepiploic artery, the short gastric artery, and the posterior gastric artery (PGA)^[1,2]. While surgeons pay a great deal of attention during upper gastric operations to the PGA, due to the technical attending point and potential for cancer metastasis to lymph nodes, their knowledge of PGA features and characteristics stems from experience only^[1,3-10]. Traditional or anatomical charts lack information on the anatomical features of the PGA^[11-14]. Therefore, the aim of the present study was to investigate features of the PGA with respect to incidence, location and size using autopsy subjects.

MATERIALS AND METHODS

Subjects

Autopsies were performed on 72 cadavers of adults with no history of abdominal operations. Of the 72 cadavers evaluated in this study, 33 were men and 39 were women, ranging in age from 45 to 98 (mean, 77.6) years. Cadavers were preserved by injection of 40 g/L formaldehyde solution.

Methods

Once the abdominal cavity was opened, the celiac trunk was identified following tissue dissection of the hepatoduodenal ligament in the direction of the pancreatic head along the common hepatic artery. The splenic artery was then carefully dissected from its origin to the hilum of the spleen and its branches, including the superior and inferior polar arteries, which were identified. Special attention was paid to the vessels of the posterior wall of the stomach. For each of these arteries, the origin, caliber, length, branching, and distribution were observed and measured directly. We determined the length of the splenic artery from the root to the hilum of the spleen

to its branching point into the superior and inferior polar arteries. Identification of the PGA was performed by experienced upper digestive tract surgeons from the Kochi Medical School.

Statistical analysis

The findings were compared by the unpaired *t*-test. *P* < 0.05 was considered significant.

RESULTS

The PGA was recognized in all cadavers. In 70 (97.2%) of 72 cadavers, the PGA branched from the splenic artery, except for 2 cadavers (2.8%). One female cadaver had two PGAs that branched from the splenic artery. The PGA originated from the root of the celiac trunk in one (1.4%) cadaver, and originated from the superior polar artery in another (1.4%). The length of the splenic artery was 11.8-18.8 (mean, 15.3) cm. Thus, there were no significant differences in the length of the splenic artery according to gender or age (Table 1).

The features of branching of the PGA from the splenic artery are summarized in Table 1. The length between the root of the splenic artery and the point of branching of the PGA ranged between 5.8-12.2 (mean, 8.4) mm, the shortest being about half the length of the longest. The length of the PGA from its origin at the splenic artery in males and females was 5.8-12.2 (mean, 9.0) cm and 6.2-10.8 (mean, 8.3) mm, respectively. The external diameter of the PGA in males and females was 1.5-3.2 (mean, 2.1) mm and 1.2-3.0 (mean, 2.0) mm, respectively. Overall, the external diameter of the PGA ranged between 1.2-3.2 (mean, 2.0) mm. There was no significant difference in the length or external diameter of the PGA with respect to gender or age.

DISCUSSION

The blood supply of the stomach is well described^[1,2]. In the upper part of the stomach, the most important nutritional blood vessel is the left gastric artery, followed by the PGA, short gastric artery, and left gastroepiploic artery. Although the PGA is one of the most important vessels supplying the upper third of the stomach the anatomical features of the PGA are not well established. Based on diagnostic imaging the incidence of the PGA is reported to range from 36% to 86%^[1,3-10]. These results are inconsistent with our previous findings using digital subtraction angiography (DSA) and multi-detector row computed tomography (MD-CT) which indicate that almost all men have a PGA^[10,15]. In the present study, the incidence of the PGA was further investigated together with features of the PGA, including location and size, using autopsy investigations by experienced upper digestive tract surgeons.

We found that all men had a PGA and the average distance between the root of splenic artery and the origin of the PGA, was 8.4 cm, which is not significantly shorter than our previous findings of 9.1 cm using MD-CT (*P* = 0.1057). The present results support our previous finding that the PGA usually (in 97.2% of humans) branches

Table 1 Features of posterior gastric artery as seen in autopsy subjects

Characteristics	<i>n</i>	Length of SA (cm)	Distance from root of SA (cm)	External diameter of PGA (mm)	<i>P</i>
Overall	70	15.3 (11.8-18.8)	8.4 (5.8-12.2)	2.0 (1.2-3.2)	0.0963
Gender					0.2274
Male	32	15.6 (12.7-18.8)	8.6 (5.8-12.2)	2.1 (1.5-3.2)	0.2462
Female	38	15.1 (11.8-18.2)	8.3 (6.2-10.8)	2.0 (1.2-3.0)	
Age (yr)					0.6897
< 80	31	15.3 (12.7-18.8)	8.5 (6.2-12.2)	2.0 (1.2-3.0)	0.4985
≥ 80	39	15.4 (11.8-17.9)	8.3 (5.8-10.1)	2.1 (1.3-3.2)	0.7876

SA: Splenic artery; PGA: Posterior gastric artery. All *P* values were non-significant between males and females, between < 80 yr and ≥ 80 yr for length of SA (cm), distance from root of SA (cm), and external diameter of PGA (mm). Data are expressed as mean (range).

from the splenic artery. According to previous reports, there are several different branching patterns of the PGA with PGA branching not only from the splenic artery but also from the celiac trunk or superior polar artery. In our previous study, using DSA and MD-CT, it was not possible to examine the relationship between the PGA and the superior polar artery. In the present study anatomical variation of the PGA was revealed by autopsy examination. In one person (1.4%) the PGA originated directly from the root of the celiac trunk and in another person (1.4%) the PGA originated from the superior polar artery.

The average external diameter of the PGA was 2.0 mm with a range of 1.2-3.2 mm, which is significantly larger than our previous finding of 1.0-1.4 mm for the internal diameter using DSA or MD-CT (*P* < 0.01). Due to its significant size, the PGA was recognized by surgeons during operation of the upper digestive tract. It is important for surgeons to be aware of the anatomical variations of the PGA, including those identified in the present study in which the PGA originated from the root of the celiac trunk or from the superior polar artery.

The incidence of adenocarcinoma of gastric cardia and/or the upper third of the stomach has recently increased in Japan as in the West^[16,17]. Of note, lymph node metastasis in gastric cancer is *via* the major lymphatic vessels in the upper third of the stomach along the left gastric artery, the PGA and the short gastric artery. Since lymph node metastasis is one of the main factors determining the prognosis of patients with gastric carcinoma, it is considered important that lymph nodes along the left gastric artery and the PGA be carefully observed and dissected when gastrectomy with regional lymphadenectomy is performed for patients with gastric carcinoma^[18-21]. In gastric carcinoma it is important to identify the PGA, not only to perform an accurate lymphadenectomy, but especially to avoid vascular complications. The high incidence of the PGA (as here reported) and its hidden origin create a significant risk of dangerous bleeding if damaged. Awareness of this anatomical structure during stomach surgery would help minimize vascular complications. Although we did not clarify the contribution of lymph nodes associated with the PGA in this case, it is likely that lymph node metastases

are associated with the PGA. Findings from the present study provide valuable information towards understanding anatomical variation of the PGA. To fully investigate the features of the PGA requires further collection and evaluation of data.

In conclusion, the present study shows that anatomical features of the PGA can be observed and evaluated efficiently by autopsy investigations and has provided valuable information on the features of the PGA useful in the planning of surgical treatment.

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S- Editor Pan BR L- Editor Zhu LH E- Editor Bi L



RAPID COMMUNICATION

Synchronous occurrence of gastrointestinal stromal tumors and other primary gastrointestinal neoplasms

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Abstract

AIM: To review clinical and pathologic features of Gastrointestinal stromal tumors (GISTs) occurring synchronously with other primary gastrointestinal neoplasms.

METHODS: Twenty-eight patients with primary GIST were treated at our institution between 1989 and 2005. Clinical and pathologic records were reviewed.

RESULTS: The gastrointestinal stromal tumor occurred simultaneously with other primary GI malignancies in 14% of all patients with GIST. The synchronous stromal tumors were located in the stomach and were incidentally found during the operation. The coexistent neoplasms were colon adenocarcinoma, gastric cancer (2 cases) and gastric lymphoma.

CONCLUSION: The synchronous occurrence of GISTs and other gastrointestinal malignancies is more common than it has been considered. The development of gastrointestinal stromal tumors and other neoplasms may involve the same carcinogenic agents.

Key words: Gastrointestinal stromal tumors; Synchronous neoplasms

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are an uncommon mesenchymal neoplasm affecting the GI tract. The synchronous occurrence of mesenchymal tumors and other primary gastrointestinal malignancies has been rarely reported in the literature^[1,2]. Most of these publications describe single case reports. We present a series of four patients, from a single institution, with GIST and a second primary neoplasm occurring synchronously. The aim of this study was to evaluate clinical and pathologic features of GISTs concomitant with other gastrointestinal malignancies.

MATERIALS AND METHODS

Patients

Between 1989 and 2005, 28 patients with primary gastrointestinal stromal tumor were treated operatively at the department of General and Gastroenterological Surgery and Nutrition, Warsaw University of Medicine. Most of the patients were women (62%). Median age of the patients at the time of presentation was 63.5 years. The primary tumor was located in the stomach (57%), small intestine (32%), large intestine (7%) and mesentery (4%). GIST was incidental in 29% of the patients. Hospital charts, operative and pathological reports were reviewed for each patient.

Methods

The histological diagnosis of all GISTs was confirmed at the department of pathology of the Warsaw University of Medicine. Specimens were fixed in 10% formaldehyde and processed routinely for paraffin embedding. 5-μm-thick sections were stained with HE. Mitoses were counted in 50 high-power fields. Malignant potential of the GISTs was stratified according to the risk categories proposed by Fletcher *et al*^[3].

Immunohistochemistry was performed using commercially available antibodies against CD117 (polyclonal, Dako, Glostrup, Denmark) and against CD34 (monoclonal, Dako, Glostrup, Denmark).

RESULTS

GIST occurred synchronously with an other gastrointestinal malignancy in four patients (14%) out of all the patients with primary gastrointestinal stromal tumor treated

Table 1 Characteristics of the patient population

No.	Age	Sex	Synchronous malignancy	Clinical presentation
1	63	F	Colon cancer	Intestinal obstruction
2	77	F	Gastric lymphoma	Abdominal pain
3	64	F	Gastric cancer	Pyloric stenosis
4	66	M	Gastric cancer	Abdominal pain, weight loss

Table 2 Pathologic features of the synchronous GISTs and other primary gastrointestinal neoplasms

No.	GIST		Synchronous gastrointestinal malignancy		
	Location	Size (cm)	Type	Location	Size (cm)
1	Anterior gastric wall (corpus)	2	Adenocarcinoma (pT4, N2, M0)	Cecum	10
2	Anterior gastric wall (fundus)	1	Lymphoma (diffuse large B cell)	Lesser curvature at the gastric angle	2
3	Anterior gastric wall (corpus)	2	Adenocarcinoma -Lauren diffuse type (pT4, N0, M0)	Antrum	5
4	Anterior gastric wall (corpus)	1	Adenocarcinoma -Lauren intestinal type (pT1, N0, M0)	Posterior wall at the gastric angle	1

Table 3 Histopathologic features of the synchronous GISTs

No.	CD117 reactivity	CD34 reactivity	Tumor size (cm)	Mitotic index	Risk category for malignant behavior ^[3]
1	+	+	2	2/50 hpf ¹	Low
2	+	+	1	0/50 hpf	Very low
3	+	+	2	2/50 hpf	Low
4	+	+	1	0/50 hpf	Very low

¹High-power fields.

in our department. Twenty-five percent of the gastric stromal tumors and 50% of the incidental GISTs were synchronous with the second gastrointestinal malignancy. The concomitant neoplasms were also primaries. All these GISTs were located in the stomach. In 75% of the cases, GIST was synchronous with other gastric malignancies, and one patient had a coexistent gastric stromal tumor and colorectal cancer. The patients presented clinically with abdominal pain, weight loss or partial intestinal obstruction. All the patients required operation due to the symptoms as a result of the tumor and GIST synchrony. The stromal tumor was always an incidental finding during the operation. The clinical characteristics of the patient population are shown in Table 1.

The synchronous gastric tumors were located in the different parts of the stomach. The average size of GIST was 1.5 cm. Morphologically, the 1-cm GISTs were a whitish, smooth and firm intramural nodule that was slightly elevated above the stomach wall. Larger GISTs were irregular, soft and a lobulated mass attached to the stomach (Figure 1). In one of the patients, the GIST was initially considered intraoperatively as a metastatic lymph node (Case 3). However, the intraoperative frozen section revealed GIST tissue. The pathologic features of the synchronous tumors are summarized in Table 2.

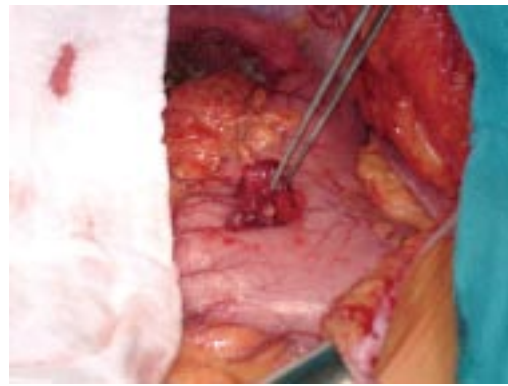


Figure 1 Intraoperative view of the gastric stromal tumor that was synchronous with colon adenocarcinoma (Case 1).

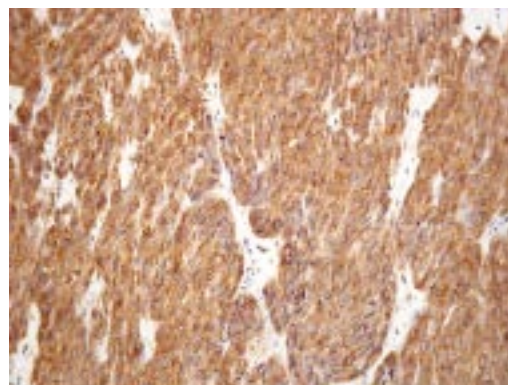


Figure 2 Strongly positive CD117 immunostaining in the synchronous gastric stromal tumor (Case 2).

Total ($n = 1$) or subtotal gastrectomies ($n = 2$) were performed in the patients with gastric malignancy. In these cases, the concomitant GIST was located within the resection margins. The patient with both colon cancer and gastric stromal tumor had a right hemicolectomy and additionally local resection of the stromal tumor.

The synchronous gastrointestinal stromal tumors were uniformly CD117 and CD34 positive (Figure 2) and could be classified as low and very low risk tumors for malignant potential. The histopathologic features of the synchronous GISTs are shown in Table 3.

DISCUSSION

Gastrointestinal stromal tumors are uncommon mesenchymal neoplasms occurring within the abdominal cavity. Most GISTs are located in the stomach and small intestine^[4]. GISTs usually develop in a sporadic fashion. However, familial occurrence has also been reported^[5]. In patients with Carney's triad, GISTs may develop together with pulmonary chondroma and extra-adrenal paraganglioma^[6]. Although 10% of the patients enrolled in the Polish GIST Clinical Registry had a second neoplasm^[7], these were usually metachronous and occurred earlier than the GIST. Slightly above 30 cases of the synchronous occurrence of mesenchymal tumors (including GIST) and other gastrointestinal malignancy have been reported in the literature^[1]. Most of these

publications describe gastric stromal tumors synchronous with another gastric malignancy. We also observed such gastric tumor association in our group. In one of our patients, GIST occurred simultaneously with colon cancer. To our knowledge, only a few reports of such tumor co-occurrence have been published in the literature^[8].

GISTs have been reported to occur synchronously with adenocarcinoma, lymphoma and carcinoid^[1,9]. Similar to other authors, GIST was most frequently synchronous with adenocarcinoma also in our series (75%). High percentage of synchronous GISTs and other gastrointestinal tumors in our series is both surprising and difficult to explain. 14% of all the GISTs and 25% of the gastric stromal tumors developed synchronously with a second gastrointestinal malignancy. This rate of neoplasm co-occurrence is greater than twice that observed in the largest group of synchronous GISTs published by Maiorana *et al*^[1]. In their series, 11.5% of gastric GISTs (6 cases) were associated with other gastrointestinal malignancies.

Although the synchronous occurrence of GIST and other abdominal malignancy seems to be just a coincidence, the development of these tumors may involve common carcinogenic agents. For example Sugimura *et al*^[10] revealed that enteral nitrosoguanidine produces adenocarcinoma in rats. In contrast, simultaneous exposure to both nitrosoguanidine and acetylsalicylic acid causes synchronous development of both gastric cancer and leiomyosarcoma^[11]. In conclusion the synchronous occurrence of GISTs and other gastrointestinal malignancies is more common than it has been considered. The concomitant GIST is usually discovered incidentally during the operation performed because of the other malignancy. The development of GIST and other neoplasms may involve the same carcinogenic agents.

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***In vitro* and *in vivo* effects of ferulic acid on gastrointestinal motility: Inhibition of cisplatin-induced delay in gastric emptying in rats**

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INTRODUCTION

Phenolic acids are plant components ubiquitously present in many fruits, vegetables and grains. They constitute a major portion of the human daily intake of non-nutrients^[1]. There is increasing evidence of positive health benefits of the high dietary intake of such compounds. These dietary phenolic acids have demonstrated antioxidant^[2], anti-inflammatory^[3], cardioprotectant^[4] and anticancer properties^[5].

Ferulic acid, a hydroxycinnamic acid, is found in rice, wheat, barley, roasted coffee, tomatoes, asparagus, olives, berries, vegetables, citrus fruits and leaves, and many other plants. Ferulic acid exhibits strong antioxidant and anti-inflammatory activities^[6]. Moreover, it has been claimed to protect against chemotherapy-induced side effects by enhancing the natural immune defense^[7].

There are previous reports that indicate beneficial effects of phenolic acids on the gastrointestinal tract^[8] and caffeic acid may enhance intestinal motility. Green tea polyphenolics improved bowel movement regularly^[9] and softened stool consistency^[10]. Also black tea polyphenols have been shown to enhance gastrointestinal motility both *in vitro* and *in vivo*^[11].

Cancer chemotherapy causes severe nausea, vomiting and abdominal discomfort which limits its administration^[12]. Most anticancer agents slow down gastric emptying^[13-15]. Cisplatin is extensively used for management of oncological disorders, particularly of the ovary, testis, bladder, head and neck^[16]. Although effective, cisplatin is associated with many adverse drug reactions, such as renal damage, gastrointestinal dysfunction, auditory toxicity and peripheral nerve toxicity^[17]. Cisplatin produced dose-related inhibition in gastric emptying reflects an accumulation of food in the stomach^[18]. Cisplatin-induced side effects in the gastrointestinal tract may be altered by metoclopramide^[19], 5HT₃ receptor antagonists^[14,15] and antioxidants^[20].

The aim of the present study was to investigate the effect of ferulic acid on gastrointestinal tract both *in vitro* and *in vivo*. The effect of ferulic acid, in selected doses, on cisplatin-induced delay in gastric emptying in rats was also investigated. Specific 5HT₃ receptor antagonists are effective against drug-induced emesis^[21] and ondansetron has been shown to reverse the delay in gastric emptying in rats^[14], therefore ondansetron was used for comparison.

Abstract

AIM: To study the effects of ferulic acid on gastrointestinal motility both *in vitro* and *in vivo*.

METHODS: Ferulic acid induced concentration-dependent stimulation of the basal tone of isolated guinea pig ileum (2-20 μ mol/L) and isolated rat fundus (0.05-0.4 mmol/L).

RESULTS: Ferulic acid significantly accelerated the gastrointestinal transit and gastric emptying in rats in a dose-dependent manner (50-200 mg/kg, po). Cisplatin (2.5-20 mg/kg, ip) induced a dose-dependent delay in gastric emptying in rats. Pretreatment with ferulic acid dose-dependently, significantly reversed the cisplatin-induced delay in gastric emptying.

CONCLUSION: The endogenous prostaglandins (PGs) are involved in mediating the stimulant effects of ferulic acid. This effect of dietary ferulic acid may help improve other accompanying gastrointestinal symptoms such as abdominal discomfort and also may protect against emesis induced by cytotoxic drugs.

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Key words: Ferulic acid; Cisplatin; Gastric emptying; Rats

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MATERIALS AND METHODS

Chemicals

All chemicals, unless specified otherwise, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Animals

Experiments were performed using Egyptian Giza strain guinea pigs (300-500 g) of either sex and adult male Wistar albino rats (175-210 g), both bred in the animal facility of Faculty of Pharmacy, Al-Azhar University. The animals were housed under conditions of $24 \pm 2^\circ\text{C}$, 50 ± 10 relative humidity and 12 h light and dark cycles. The animals were fed with a laboratory diet manufactured by Al-Nasr Co., Cairo, Egypt, and were fasted overnight before drug administration.

In vitro experiments

Isolated rat fundus: The experiment was made according to Vane^[22]. Adult male Wistar albino rats were fasted overnight with free access to water, and then they were sacrificed. The abdomen of each animal was opened, and the entire stomach was dissected free from the abdomen and placed into a dish containing aerated Krebs solution at 37°C . The grey fundal part was separated from the pink pyloric part of each stomach. Each part was cut longitudinally to form a sheet strip of gastric fundus approximately 4 cm long and 3 mm wide (the cuts were parallel to the longitudinal muscle fibers by making alternate transverse cuts on opposite sides of the muscle). A thread was attached to each end of the preparation, and then it was suspended in a 20 mL organ bath filled with Krebs solution at 37°C and aerated with a mixture of oxygen (95%) and carbon dioxide (5%). One thread was attached to a fixed pin in the bath and the other to isotonic transducer (Washington 400 MD2C Bioscience, Sheerness, Kent, UK). The gastric fundus preparation was allowed to equilibrate for 30 min under tension of 1 g before starting the experiment. Ferulic acid at different concentrations was allowed to contact with the preparation for 6 min. Serotonin ($14 \mu\text{mol/L}$) was used at the beginning of each experiment to test the sensitivity of the tissue used. To determine the site of action of ferulic acid, a submaximal concentration (0.05 mmol/L) was tested before and after complete blockade of H_1 receptors (mepyramine $10 \mu\text{mol/L}$), muscarinic receptors (atropine sulfate $10 \mu\text{mol/L}$), 5HT_3 receptors (partially blocked by ondansetron 1 mmol/L) and PGE_2 effect (blocked by indomethacin 0.08 mmol/L).

Isolated guinea pig ileum: Experiments were done according to Perry^[23]. Guinea pigs were killed by exsanguination. The guinea pig ileum preparation was set up in a 20 mL organ bath containing Tyrode solution at 37°C gassed with 95% O_2 and 5% CO_2 . The normal tone and changes in force of contraction after different concentrations of ferulic acid ($2\text{--}20 \mu\text{mol/L}$) were measured (at a speed of recording 0.25 mm/sec) using isotonic transducer (Washington 400 MD2C Bioscience, Sheerness, Kent, UK). Histamine ($2\text{--}25 \mu\text{mol/L}$) was used to test the sensitivity of the tissue. To determine the site of action of ferulic acid, mepyramine ($0.8 \mu\text{mol/L}$), atropine sulfate ($1 \mu\text{mol/L}$), ondansetron ($5 \mu\text{mol/L}$)

and indomethacin (0.14 mmol/L) were used to mediate blockade of H_1 receptors, muscarinic receptors, 5HT_3 receptors and the PGE_2 effect, respectively.

In vivo experiments

Intestinal transit rate in rats: Gastrointestinal transit was measured in the charcoal propulsion test^[24]. The test compound (50, 100 or 200 mg/kg) and saline (control) were administered orally. Rats were distributed into groups (8 each): group 1 received 1 mL saline (control); groups 2-4 were treated with ferulic acid in 3 doses (50, 100 or 200 mg/kg , oral); group 5 was given indomethacin (0.75 mg/kg , ip)^[11]; and Group 6 received indomethacin (0.75 mg/kg , ip) 50 min after oral administration of ferulic acid (100 mg/kg). One hour after ferulic acid administration, each rat was orally administered 1 mL charcoal meal (5% activated charcoal suspended in 10% aqueous tragacanth). Rats were killed 30 min later by cervical dislocation. The extent of charcoal propulsion in the small intestine was measured (distance traveled by the charcoal head from the pylorus as well as total length of the small intestine). Intestinal transit rate% = distance traveled by charcoal head/length of the small intestine $\times 100$.

Gastrointestinal emptying in rats: The rate of gastric emptying of the non-nutrient semisolid meal was determined according to the method described previously^[25]. Male Wistar rats were fasted overnight with water *ad libitum*. Briefly, a test meal (0.05% phenol red in a 1.5% aqueous methylcellulose solution) was given (1.5 mL/rat) by gastric tube. Thirty minutes after the meal was given, the animals were sacrificed. The cardia and the pylori were clamped, the stomach was removed out, homogenized with its contents in 100 mL of 0.1 mol/L NaOH. Proteins (in 5 mL homogenate) were precipitated with 0.5 mL of trichloroacetic acid (20% w/v) and centrifuged. The supernatant was mixed with 4 mL of 0.5 mol/L NaOH and absorbance of the sample was read at a wavelength of 560 nm. Phenol red was recovered from the stomach of rats killed immediately after administration of the methylcellulose meal served as the standard stomach. The percentage of gastric emptying was calculated from the following formula: Gastric emptying (%) = $1 - (\text{amount of phenol red recovered from the test stomach} / (\text{average amount of phenol red recovered from the stomach}) \times 100$.

Activity is expressed as the percent change of gastric emptying in treated rats versus controls.

Tested compounds

Effect of ferulic acid: Ferulic acid at three different doses (50, 100 or 200 mg/kg) was orally administered 30 min before the test meal.

Effect of cisplatin: To assess the dose dependency of cisplatin-induced delay in gastric emptying and to standardize the dose that would be more suitable to study the effect of ferulic acid pretreatment, cisplatin was administered at different doses of 2.5, 5, 10 and 20 mg/kg , ip, 30 min before the test meal administration.

Effect of ferulic acid on cisplatin-induced delay in gastric emptying: Effect of ferulic acid (100 mg/kg) was investigated on the delay of gastric emptying induced by cisplatin 10 mg/kg , ip. Ferulic acid was given 20 min before cisplatin. The effect of ferulic acid was compared with

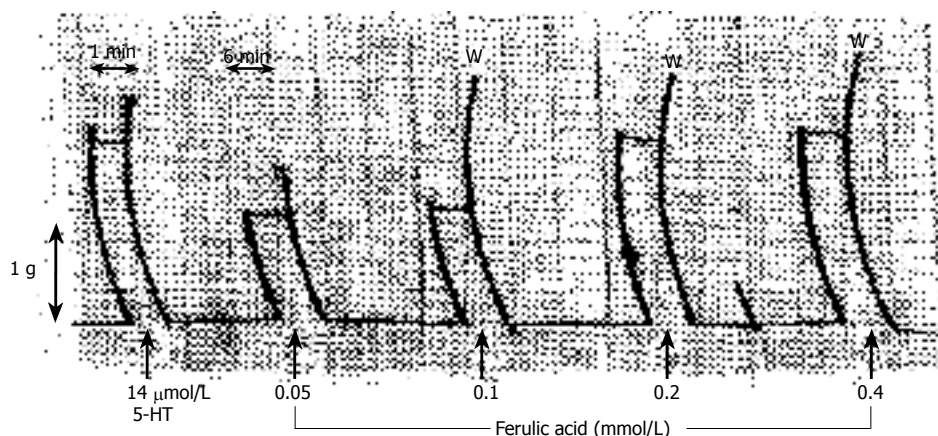


Figure 1 Effect of ferulic acid on isolated rat fundus. Fundic strip was suspended in oxygenated Krebs solution at 37°C. The 1 min and 6 min scales represent the speed of recording and the 1-g scale represents the calibration tension and W is a wash.

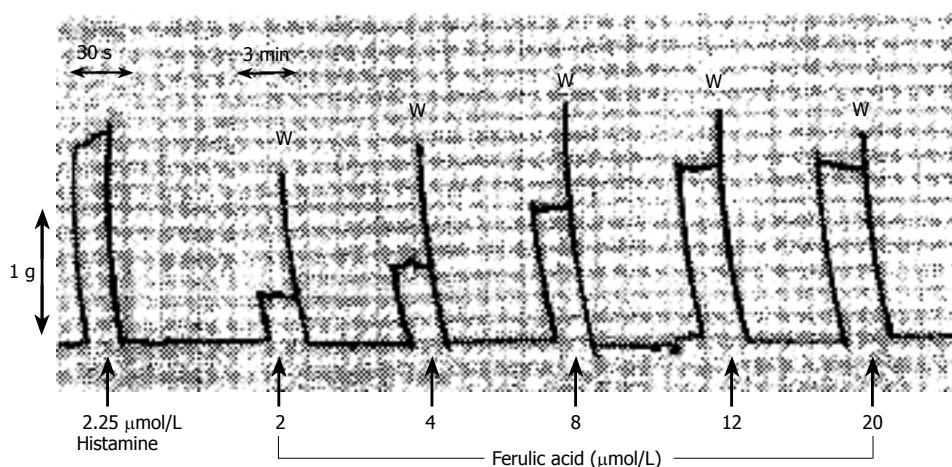


Figure 2 Effect of ferulic acid on isolated guinea pig ileum. Ileum was suspended in oxygenated Tyrode solution at 37°C. The 30 s and 3 min scales represent the speed of recording and the 1-g scale represents the calibration tension and W is a wash.

the 5HT₃ receptor antagonist ondansetron administered at a dose of 3 mg/kg, po, 30 min before cisplatin administration.

Statistical analysis

The results are expressed as the mean \pm SE. One way analysis of variance (ANOVA) followed by Bonferroni test for multiple comparisons was used for statistical comparison. *P* values less than 0.05 were considered statistically significant.

RESULTS

Isolated rat fundus

Ferulic acid (0.05-0.4 mmol/L) produced concentration-dependent contractions of isolated rat fundus (Figure 1). These contractions were not affected by blockade of muscarinic receptors by atropine sulfate (10 μ mol/L) or by the histaminergic receptor with mepyramine (10 mmol/L). On the other hand, ondansetron (1 mmol/L) did not affect the contraction. Complete blockade of PGE₂ by indomethacin partially decreased the contractile response due to ferulic acid.

Guinea pig ileum

Ferulic acid (2-20 μ mol/L) induced a significant ($P < 0.05$) increase in the height of contraction of isolated guinea pig ileum with a maximum concentration of 20 μ mol/L (Figure 2). A submaximal concentration (4 μ mol/L) of ferulic acid was used to investigate the site of its stimulant

effect. Complete blockade of either H₁ or muscarinic receptors by mepyramine or atropine did not affect the response of ileum to ferulic acid. Also, partial blockade of 5HT₃ receptors by ondansetron failed to prevent the stimulant effect. The stimulant effect of ferulic acid was partially inhibited by indomethacin (0.14 mmol/L). This stimulant effect may be mediated at least partly *via* stimulation of PGE₂ receptors.

Intestinal transit rate in rats

Ferulic acid significantly induced a dose-dependent increase in the distance traveled by charcoal meal in the gut of rats at 50, 100 or 200 mg/kg. The facilitatory effect of ferulic acid (100 mg/kg) was significantly reduced after pretreatment with indomethacin. Indomethacin alone significantly reduced the intestinal transit rate as compared with the control group (a decrease of 12.1%). Ferulic acid (100 mg/kg) significantly increased the intestinal transit rate of the indomethacin-treated group (by 27.5% compared with the indomethacin group and 12.1% compared with the control group) (Table 1).

Gastric emptying rate

Effect of cisplatin: Intraperitoneal administration of cisplatin dose-dependently inhibited the gastric emptying rate of the phenol red methylcellulose meal in rats. After administration of cisplatin 2.5, 5, 10, and 20 mg/kg, the gastric emptying was 41% \pm 3.1%, 25% \pm 2.2%, 15% \pm 1.2% and 6% \pm 0.4%, respectively as compared with the control 61% \pm 3.0%.

Table 1 Effect of ferulic acid on gastrointestinal transit rate of a semisolid charcoal meal in rats

Treatment	Dose	Transit rate (% \pm SE)	% change of control
Saline (control)	1 mL, oral	58 \pm 1.5	0.0
Ferulic acid	50 mg/kg, oral	73 \pm 3.1	25.9
	100 mg/kg, oral	85 \pm 2.1	46.6
	200 mg/kg, oral	91 \pm 3.2	56.9
Indomethacin	0.75 mg/kg, ip	51 \pm 2.0	- 12.1
Indomethacin + ferulic acid	0.75 mg/kg, ip + 100 mg/kg, po	65 \pm 3.3	12.1

Compounds were orally administered 60 min before the test meal (10 mL/kg body weight of 5% charcoal in 5% gum tragacanth). $P < 0.05$ vs control group or indomethacin group, using ANOVA followed by Bonferroni test for multiple comparisons.

Effect of ferulic acid: Ferulic acid induced a dose-dependent (50-200 mg/kg, po) significant increase on the gastric emptying rate. The percentage increases at 50, 100 and 200 mg/kg were 22.4%, 48.3% and 70.7%, respectively.

Effect of 5HT₃ receptor antagonist ondansetron: Ondansetron 3 mg/kg, po when administered 30 min before cisplatin significantly increased the gastric emptying rate to 49% \pm 2.5% compared with cisplatin (10 mg/kg, ip) group 15 \pm 1.2 (Figure 3).

Effect of ferulic acid on cisplatin-induced delay in gastric emptying: Pretreatment with oral ferulic acid at doses of 100 and 200 mg/kg, increased gastric emptying to 30% \pm 1.7% and 51% \pm 3.1%, respectively as compared to cisplatin (10 mg/kg) alone. The reversal of delayed gastric emptying was statistically significant at both doses ($P < 0.05$) (Figure 3).

DISCUSSION

The present data revealed the prokinetic effect of ferulic acid *in vitro* and *in vivo*. Ferulic acid showed a significant dose-dependent stimulation of the basal tone of isolated rat fundus and isolated guinea pig ileum. The findings have provided evidence for the involvement of endogenous PGs in mediating the stimulant effect of ferulic acid. Both the gastric and intestinal mucosa synthesize large amounts of PGs^[26]. PGE₂ was shown to contract longitudinal smooth muscles^[27] and relax the circular smooth muscle of the intestine^[28], while indomethacin could relax the longitudinal intestinal smooth muscles^[29] and contract the circular muscles due to inhibition mostly of PGE₂ and PGI₂^[30]. The antagonistic effect of ferulic acid on gastrointestinal transit time by indomethacin indicates a role of PGs in the mechanism of action of ferulic acid on gastrointestinal motility.

The effect of ferulic acid on peristalsis and gastric emptying further supports the gastrokinetic effect of ferulic acid. The observation that indomethacin could reduce the facilitatory effect of ferulic acid further indicates the partial PG involvement in the mechanism of action of ferulic acid on gastrointestinal motility.

Phenolic acids were reported to inhibit lipoxygenase activity^[31], leukotrienes^[32] and thromboxan^[33] biosynthesis,

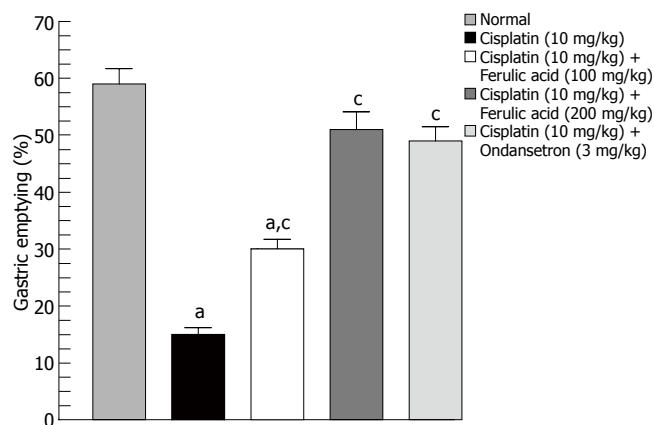


Figure 3 Effect of ferulic acid and ondansetron on cisplatin-induced delay in gastric emptying in rats (mean \pm SE, $n = 8$, $^{a,c}P < 0.05$, vs control or cisplatin group, respectively).

so ferulic acid may shunt the arachidonic acid metabolism to the cyclooxygenase pathway especially towards PGs biosynthesis direction.

There is a possibility, although not investigated in the current study, that the gastrokinetic activity of ferulic acid may be partially mediated *via* interference with nitric oxide (NO) production, and NO plays a key role in regulation of gastrointestinal motility by its smooth muscle relaxing and vasodilating activity^[34]. Based on this assumption, Soliman and Mazzio^[35] and Son *et al*^[36] reported that caffeic acid significantly inhibited NO production probably *via* inhibition of NO synthase gene expression.

Further studies on the mechanism of action of ferulic acid on gastrointestinal motility may be helpful in determining the therapeutic values of ferulic acid in gastrointestinal motor disorders.

Cisplatin plays an important role in the treatment of malignant diseases^[14]. It causes severe nausea and vomiting, accompanying gastrointestinal symptoms such as abdominal discomfort in the patients^[37]. Cisplatin when given intravenously in dogs causes abnormal myoelectric activity in the antrum and small intestines^[38], abdominal distension and delayed gastric emptying in rats^[14,20].

In the present study in rats, ip administration of cisplatin dose-dependently inhibited the gastric emptying rate after a non-nutrient meal. These results are consistent with the finding that cisplatin causes a delay in gastric emptying in rats^[14,15,20]. This effect may be due to the release of 5HT from the mucosal enterochromaffin cells in the gastrointestinal tract. The 5HT releases the peripheral 5HT₃ receptors on the vagal afferent fibers and causes relaxation of the stomach possibly leading to delay in gastric emptying^[37]. Also, cisplatin-induced free radical generation in the intestine^[39] may lead to subsequent release of 5HT^[40] which may act on peripheral 5HT₃ to the relax the stomach and lead to the delay in gastric emptying.

The inhibitory action of cisplatin on gastric emptying was significantly reversed by pretreatment with ferulic acid. The effect of ferulic acid was dose-dependent. The beneficial effect of ferulic acid could be attributed at least partly to its stimulant effect on gastrointestinal tract and its antioxidant effect. Ferulic acid, as one of the phenolic acids, has demonstrated strong antioxidant properties

as its structure dependent hydrogen-donating abilities and its propensity for nitrate make these compounds powerful scavengers of reactive oxygen species and reactive nitrogen species^[2]. Ferulic acid has been shown to protect gastrointestinal organs against carcinogenesis^[41] and antioxidants may prevent cisplatin-induced delay in gastric emptying in rats. In conclusion, ferulic acid inhibits the cisplatin-induced delay in gastric emptying in rats. This agent may be useful in reducing cisplatin-induced emesis and improve gastrointestinal symptoms such as abdominal discomfort induced by cytotoxic agents.

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RAPID COMMUNICATION

Differential reactivity of mouse monoclonal anti-HBs antibodies with recombinant mutant HBs antigens

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(M→T) were found to affect reactivity of these mAbs.

CONCLUSION: Our findings could have important implications for biophysical studies, vaccination strategies and immunotherapy of hepatitis B virus (HBV) mutants.

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Key words: Hepatitis B surface antigen; Hepatitis B virus; Mutant; Epitope mapping; Vaccination; Monoclonal antibody

Roohi A, Yazdani Y, Khoshnoodi J, Jazayeri SM, Carman WF, Chamankhah M, Rashedan M, Shokri F. Differential reactivity of mouse monoclonal anti-HBs antibodies with recombinant mutant HBs antigens. *World J Gastroenterol* 2006; 12(33): 5368-5374

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Abstract

AIM: To investigate the reactivity of a panel of 8 mouse anti-hepatitis B surface antigen (HBsAg) monoclonal antibodies (mAbs) using a collection of 9 recombinant HBsAg mutants with a variety of amino acid substitutions mostly located within the "a" region.

METHODS: The entire HBs genes previously cloned into a mammalian expression vector were transiently transfected into COS7 cells. Two standard unmutated sequences of the ayw and adw subtypes served as controls. Secreted mutant proteins were collected and measured by three commercial diagnostic immunoassays to assess transfection efficiency. Reactivity of anti-HBs mAbs with mutated HBsAg was determined by sandwich enzyme-linked immunosorbent assay (ELISA).

RESULTS: Reactivity of anti-HBs mAbs with mutated HBsAg revealed different patterns. While three mutants reacted strongly with all mAbs, two mutants reacted weakly with only two mAbs and the remaining proteins displayed variable degrees of reactivity towards different mAbs. Accordingly, four groups of mAbs with different but overlapping reactivity patterns could be envisaged. One group consisting of two mAbs (37C5-S7 and 35C6-S11) was found to recognize stable linear epitopes conserved in all mutants. Mutations outside the "a" determinant at positions 120 (P→S), 123(T→N) and 161

INTRODUCTION

In spite of the progress made in vaccine development, hepatitis B virus (HBV) infection remains a major health care problem worldwide. Upon exposure to HBV, 5%-10% of healthy adults fail to mount a protective antibody response and become chronically infected^[1]. A similar proportion of healthy individuals do not respond to HB vaccination^[2,3]. Lack of response has largely been attributed to parameters associated to the host immune system, including defective antigen presentation^[4,5], diminished Th1/Th2 response^[6,7], restricted HBsAg-specific T- and/or B-cell repertoires^[8,9] and HLA-linked B-cell deletion or suppression^[10,11]. Viral factors, however, particularly evolving mutant variants may also play an important role. The unique replication cycle of HBV^[12] provides much opportunity for mutations to appear^[13]. In addition, immune response is considered as a synergistic pressure for emergence of these mutants^[13,14]. Amino acid substitutions resulting from such mutations may affect the structure of hepatitis B surface antigens (HBsAg) that consist of three overlapping molecules, namely large (L), middle (M) and small (S) proteins^[13].

The small protein, traditionally known as HBsAg, contains the immunodominant "a" determinant shared by all serotypes and genotypes of HBV, together with

serotypic epitopes determining the four major subtypes of HBV, that is *dm*, *ym*, *dr* and *yr*^[15]. The “a” determinant (amino acids 124-147) is located within a large antigenic area referred to as the major hydrophilic region (MHR). Tertiary structure of this area is crucial for its recognition by the immune system. Hence, mutations within this area may allow emergence of escape mutants with reduced binding affinity to HBsAg-specific antibodies. Such mutants may also skip identification by antibody-based diagnostic assays^[16,17].

Monoclonal antibodies (mAbs) against HBsAg are widely used in diagnostic immunoassays^[18,19]. However, these assays may not detect all cases of infection with variant viruses carrying mutations in some critical positions of the MHR^[16,18,20,21]. Emergence of mutant HBV variants due to the escape from immunological pressure, either following immunoprophylaxis with polyclonal or monoclonal anti-HBs antibody and/or vaccination with HBsAg, may hinder detection of HBsAg by the current monoclonal antibody-based immunoassays. Such mutations, predominantly accumulated within the “a” determinant, could also result in breakthrough infection in a number of vaccinated subjects^[22,23]. These mutants will have selective advantage for propagation and vertical or horizontal transmission to vaccinated individuals^[16,24].

Recent reports of accelerated accumulation of “a” determinant mutants in vaccinated children following an universal vaccination program^[25,26], together with a high prevalence of “a” determinant mutations in natural HBsAg mutants collected from unvaccinated carriers^[27], have brought serious concerns to the current vaccination policy. This has prompted the Viral Hepatitis Prevention Board of WHO to consider development of strategies to prepare for eventual increase of mutant viruses and to recommend establishment of an independent global network for appropriate monitoring of such mutants^[28,29].

In the present study, we have characterized eight murine HBsAg-specific mAbs as potential tools for detection of HBsAg mutants. The results presented in this paper and similar papers reported previously^[20,30,31] will help to identify the most influential mutations relevant to vaccine escape and immunodetection failure.

MATERIALS AND METHODS

Monoclonal antibodies

Eight murine mAbs were raised against a recombinant wild-type hepatitis B surface antigen (rHBsAg) of the adw subtype (Heberbiovac, Heberbiotec, Cuba) as described elsewhere^[19]. Characteristics of these mAbs are summarized in Table 1.

Production, purification and biotinylation of polyclonal anti-HBs antibody

White New Zealand rabbits were administered four injections with 10 µg of standard rHBsAg (adw) at two-week intervals. The first dose was given in complete Freund's adjuvant (Sigma, USA), whereas other doses were administered in incomplete Freund's adjuvant (Sigma, USA). Following serum titration of anti-HBs antibody

Table 1 Characteristics of monoclonal anti-HBs antibodies

Clone	Isotype	Kaff (M ⁻¹)	Recognized epitope
34D7-S3	IgG1	5.24 × 10 ⁸	Conformational
33D7-S4	IgG1	3.27 × 10 ⁸	Conformational
38F3-S6	IgG1	3.75 × 10 ⁸	Conformational
37C5-S7	IgG1	4 × 10 ⁸	Linear
35C6-S11	IgG1	2.87 × 10 ⁸	Linear
35G9-S15A	IgG1	1.73 × 10 ⁸	Conformational
47G3-S15C	IgM	NI	Conformational
31D4-H12	IgG1	2.2 × 10 ⁸	Linear

NI: Not identified.

Table 2 Mutations and subtype of recombinant mutant HBs antigens expressed in COS7 cells

Clone	Mutations	Subtype
Gly D	Standard sequence	adw
Gly y	Standard sequence	ayw
91-4696	S113T/T143S	adw
SA6	Q129R/G130N/A166V	adw
SA7	M133T	ayw
1056	P120S/S143L	ayw
PA17D	D144E/multiple mutations	NI
M5	Y100S/T118V/R122K/M133I/ Y134N/P142S/S143L/G145K	ayw
BA2.4	Y100C/P120T	ayw
SA4	M133T/y161F	adw
BA3.2	T123N/C124R	ayw

NI: Not identified.

from immunized rabbits, hyperimmune sera were collected and purified using a column of rHBsAg coupled to CNBr-activated sepharose-4B (Amersham Biosciences, Sweden). Purified antibody was biotinylated using biotinyl N-hydroxysuccinimide (BNHS) ester (Sigma) as previously described by Bayer *et al.*^[32], with some modifications. Briefly, IgG (1 mg/mL) was dialyzed overnight in sodium bicarbonate (pH 8.5). BNHS ester freshly dissolved in dimethyl sulfoxide (Merck, Germany) at 1 mg/mL was added to predialyzed IgG at a 1:10 (v/v) ratio and incubated at 25°C for 4 h. The mixture was then dialyzed overnight against 0.1 mol/L Tris-HCl (pH 7.4) dispensed into small aliquots and stored at -20°C to avoid frequent freezing and thawing.

Preparation of expression plasmid clones encoding HBsAg mutants

Plasmid DNA (pRK5) of nine HBsAg variants and two unmutated standard adw and ayw isolates (Table 2) were constructed as described previously^[20]. Plasmid DNA was propagated by transformation of *E. coli* strain DH5α (CinnaGen, Iran) by electroporation^[33]. In brief, appropriate amount (depending on the clone) of plasmid DNA was mixed with electrocompetent cells, incubated on ice and subsequently pipetted into a cold electroporation

cuvette (Bio-Rad, USA). Pulse of electricity was delivered and super optimal catobolite (SOC) medium was added immediately to electroporated cells. Following 2 h incubation at 37°C, different volumes of electroporated cells were plated onto LB agar (Sigma) containing 75 µg/mL ampicillin (Sigma). Electrocompetent cells without DNA were used as a negative control. Transformed colonies were selected, tested and cultured in LB broth containing 75 µg/mL ampicillin. Plasmid DNA was subsequently purified using G1AGEN plasmid extraction kit (QIAGEN, USA).

Transfection of expression plasmids

COS7 cells (NCBI C143) provided by National Cell Bank of Iran (Pasteur Institute of Iran, Tehran) were cultured in RPMI-1640 (Gibco, USA) containing 100 mL/L heat-inactivated fetal bovine serum (Biochrom, Germany), penicillin (100 IU/mL) and streptomycin (100 µg/mL). Cells were transiently transfected using Lipofectamin 2000 (Invitrogen, USA). The amount of viral DNA was adjusted in all experiments to 0.8 µg/well in a 24-well plate (Nunc, Denmark). A subconfluent monolayer of COS7 cells was washed with growth medium without antibiotics and 500 µL of Opti-MEM I Reduced Serum Medium (Invitrogen) was added. Plasmid DNA was diluted in 50 µL of Opti-MEM I. Then 2 µL Lipofectamin 2000 pre-diluted in 50 µL of Opti-MEM I was added to diluted DNA and kept at room temperature for 20 min. The complex was added to the cells and the cells were incubated at 37°C in a humidified atmosphere containing 50 mL/L CO₂. Following a 2 h incubation, 500 µL of complete medium was added and culture supernatant was harvested 48-96 h later. pRK5 plasmid with or without the insert carrying a standard sequence of HBV DNA [adw (Gly D) and ayw (Gly Y) subtypes] was used as negative and positive controls, respectively.

Commercial HBsAg detection kits

Supernatants of transfected cells were tested for the presence of HBsAg using three different sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's guidelines. The Bioelisa HBsAg colour kit (Biokit, Spain) employs polyclonal Ab as the capture (coating) layer, whereas the Hepanostika HBsAg Uni-Form II (BioMerieux, The Netherlands) and the ETI-MAK-4 (Diasorin, Italy) kits employ mAb as the coating Ab. All three kits contain peroxidase-conjugated polyclonal anti-HBs antibody as detector.

Determination of reactivity of anti-HBs mAbs with HBsAg mutants by sandwich ELISA

A panel of eight mAbs was used in this study. Monoclonal anti-HBs Abs were dissolved in phosphate-buffered saline (0.15 mol/L PBS, pH 7.2) at a final concentration of 10 µg/mL. The wells of 96-well flat-bottom microtiter plates (Maxisorp, Nunc, Denmark) were coated with anti-HBs Ab (100 µL/well) and incubated for 90 min at 37°C. The wells were then washed three times with PBS and plates were blocked with PBS containing 30 g/L skim milk (Merck, Germany) for 90 min at 37°C. After washing with

Table 3 Reactivity of mutant HBs antigens secreted by transfected cells as determined by three commercial ELISA kits

Expressed HBsAg	Bioelisa		ETI-MAK-4		Hepanostika		
	A ₄₅₀	Index	A ₄₅₀	Index	A ₄₅₀	Index	Conc.
Gly D	> 3	> 5.5	2.9	5.1	> 3	> 11	> 200
Gly Y	2.97	5.5	2.97	5.2	> 3	> 11	> 200
91-4696	> 3	> 5.5	2.87	5	> 3	> 11	> 200
SA6	2.96	5.5	2.74	4.8	> 3	> 11	> 200
SA7	> 3	> 5.5	2.87	5	> 3	> 11	> 200
1056	1.5	2.8	1.8	3.2	2.32	8.6	162
PA17D	1	1.8	2.7	4.7	> 3	> 11	> 200
M5	1.59	2.9	0.74	1.3	1.39	5.1	92
BA2.4	1.66	3.1	2.08	3.6	2.33	8.6	162
SA4	1.67	3.1	1.34	2.3	2.12	7.8	148
BA3.2	0.54	1	0.65	1.1	1	3.7	62
pRK5	0.17	0.31	0.08	0.15	0.1	0.37	0
Medium	0.18	0.33	0.11	0.19	0.1	0.37	0

Results are expressed as absorbance (A) measured at 450 nm, index (ratio of absorbance of mutant sample to absorbance obtained for 1.25 ng/mL of standard adw HBsAg) and concentration (Conc.) presented as ng/mL. Concentration was measured only by the Hepanostika kit using the standard adw HBsAg to construct the standard curve.

PBS containing 0.5 mL/L Tween 20 (PBS/T, Sigma), 100 µL of supernatant of the transfected cells was added to the plates. Following incubation at 37°C for 90 min, the plates were washed and filled with appropriate dilution of biotinylated-rabbit anti-HBs Ab. After incubation for 90 min and washing, appropriate dilution of peroxidase-conjugated streptavidin (Sigma) was added and the reaction was then revealed with tetramethylbenzidine (TMB, Sigma) substrate. Finally, the reaction was stopped with 200 mL/L H₂SO₄ and the absorbance (A₄₅₀) was measured by a multiscan ELISA reader (Organon Teknika, Bostel, The Netherlands) at 450 nm.

RESULTS

Detection of expressed HBsAg mutants by commercial ELISA kits

Transfection efficiency of all expressed HBsAg clones, including nine mutants and two unmutated standard sequences, was measured using 3 commercial diagnostic kits. To obtain a comparable index in each assay, absorbance (A₄₅₀) of each sample was represented as the ratio to absorbance obtained for 1.25 ng/mL of a purified recombinant unmutated HBsAg of the adw subtype (immunogen) (Table 3). All expressed HBsAg proteins were detectable by all three assays, albeit at different levels. While the index value for 91-4696 was similar to the standard sequences (Gly D and Gly Y), BA3.2 mutant showed the lowest index using all three assays, significantly lower than the standard sequences. Overall, the Hepanostika diagnostic kit gave higher absorbance and relative indexes implying better sensitivity compared to the other two kits. This kit was subsequently used to quantify the concentration of secreted HBsAg in culture supernatants of transfected cells by extrapolation from known input of the standard adw HBsAg (Table 3).

Table 4 Reactivity patterns of monoclonal anti-HBs antibodies with expressed HBs antigen mutants

Monoclonal antibody	GlyD	GlyY	91-4696	SA6	SA7	1056	PA17D	M5	BA2.4	SA4	BA3.2
34D7-S3	++	++	++	++	++	+	-	-	+	+	-
33D7-S4	++	++	++	++	++	+	-	-	+ ^w	+	-
38F3-S6	+++	+++	+++	+++	+++	+++	+ ^w	-	-	+	-
37C5-S7	+++	+++	+++	+++	+++	+ ^w	++	+++	++	+ ^w	+
35C6-S11	+++	+++	+++	+++	+++	+ ^w	+++	+	+	+ ^w	+ ^w
35G9-S15A	+++	+++	+++	+++	+++	-	-	-	+ ^w	+ ^w	-
31D4-H12	++	++	++	++	++	-	-	-	+ ^w	+ ^w	-
47G3-S15C	+++	+++	+++	+	+	-	-	+	+	-	-

Results are expressed as ratio of absorbance (A) of a given mutant to that obtained for 1.25 ng/mL of standard recombinant adw HBsAg and presented as :
 -: ≤ negative control (pRK5 plasmid); +^w: > negative control < 1; +: 1-3; ++: 3-6; +++: > 6.

Table 5 Cross inhibition of monoclonal antibodies binding to HBs antigen

Unlabeled mAbs	HRP-conjugated mAbs						
	34D7-S3	33D7-S4	38F3-S6	37C5-S7	35C6-S11	35G9-S15A	31D4-H12
34D7-S3	4	4	4	2	3	4	1
33D7-S4	3	4	4	2	4	3	2
38F3-S6	3	3	4	1	3	4	2
37C5-S7	1	1	1	4	1	2	3
35C6-S11	2	4	4	2	4	3	2
35G9-S15A	4	3	3	1	3	3	2
47G3-S15C	3	3	3	1	3	3	2
31D4-H12	2	1	1	3	1	2	3

1: 0%-20% inhibition; 2: 20%-50% inhibition; 3: 50%-80% inhibition; 4: 80%-100% inhibition.

Reactivity patterns of anti-HBs mAbs to the expressed HBsAg mutants

Immunoscreening was carried out on supernatants from cells transfected with all constructs using the mAbs as the coating layer. Supernatants of untransfected cells or cells transfected with pRK5 plasmid vector alone were used as background (blank) and negative control, respectively. The results are presented in Figure 1 and summarized in Table 4. While the standard sequences (Gly Y and Gly D) and 3 mutant proteins (91-4696, SA6 and SA7) were detectable by all eight mAbs, only two of the mAbs (37C5-S7 and 35C6-S11) reacted with BA3.2 having two mutations at positions 123 and 124. The remaining mutants displayed different reactivity patterns. Cross-inhibition studies were performed to find out whether binding of an antibody could inhibit binding of other mAbs to their epitopes, enabling identification of overlapping epitopes recognized by different mAbs. The results illustrate different inhibitory patterns, partly compatible with those obtained from the direct binding assays (Table 5).

DISCUSSION

In the present study, eight mAbs raised to a recombinant unmutated HBsAg of the adw subtype were tested for their bindings to a panel of nine mutants HBsAg. We have recently demonstrated that these mAbs recognize different conformational or linear epitopes common to adw, ayw and adr subtypes, supposedly the “a” determinant^[19].

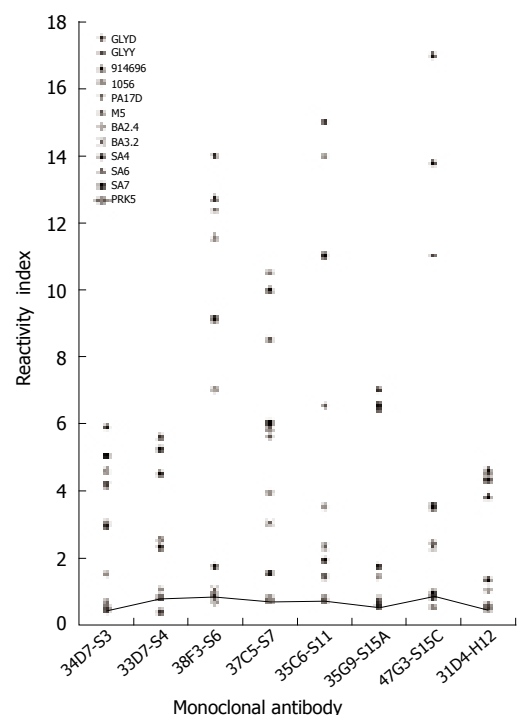


Figure 1 Reactivity of mouse monoclonal anti-HBs antibodies with recombinant HBs antigen mutants. Reactivity is expressed as relative index defined as the ratio of absorbance (A) of a given mutant to the absorbance obtained for 1.25 ng/mL of the standard adw HBs antigen. The horizontal line represents the results obtained for pRK5 plasmid without insert used as the negative control.

This determinant is conserved in all subtypes of HBV and confers a protective antibody response to HBV infection by all subtypes^[34,35]. Vaccination of healthy individuals with recombinant HBsAg has also been shown to induce an antibody response restricted to the “a” determinant^[36]. Although the structure of the “a” determinant has not been thoroughly characterized and seems to encompass a large number of amino acid residues common to all subtypes of HBV, an important region of this determinant lies between residues 124 and 147. This region is a part of a highly hydrophilic area located between residues 100 and 160. Presence of 5 cysteine residues at positions 124, 137, 138, 139 and 147 creates at least 2 loops protruding from the surface of

HBsAg as highly immunogenic epitopes. The majority of mAbs produced against HBsAg have been shown to recognize conformational epitopes within one of these loops^[16,17,30,31]. The tertiary structure created by these disulphide bonds is critical for antigenicity of HBsAg and replacement of cysteine residues at positions 124 and 147 with serine destroys its antigenicity^[37], demonstrating the conformational nature of the epitopes. Five of our mAbs reacted with conformational epitopes (Table 1), which were destroyed upon treatment of the molecule with a reducing agent, such as 2-ME.

Both type and position of substituted amino acids within the “a” determinant seem to be equally important. This is clearly reflected in the reactivity patterns of our mAbs. Thus three of the mutants including SA7, SA6 and 91-4696 were strongly reactive with all our mAbs. The reactivity indexes obtained for some of these mutants, particularly 91-4696, were similar to/or higher than those obtained for the two standard sequences (Figure 1). They also displayed strong reactivity with the three commercial diagnostic kits employed in this study. Similar reactivity patterns have already been reported for these three mutants, using a variety of commercial diagnostic kits and HBsAg-specific mAbs^[20,30]. Altogether six replacement mutations, including S113T/T143S (91-4696), Q129R/G130N/A166V (SA6) and M133T (SA7), were identified in these three mutants, none of which seemed to be crucial for HBs antigenicity. Of the two substitutions occurring at positions 113 and 143 in the 91-4696 mutant, the latter is more relevant to antigenicity of the “a” determinant. However, replacement of threonine with serine at this position has also been observed in sequences from a number of unmutated isolates of HBV deposited in the GenBank. This may explain the preserved binding activity of this mutant with all our mAbs. Surprisingly, replacement of serine with leucine at the same position in another mutant (1056) resulted in either substantial reduction or complete loss of reactivity towards most of the mAbs. Three of the mAbs (35G9-S15A, 31D4-H12 and 47G3-S15C) were completely negative and two mAbs (37C5-S7, 35C6-S11) were weakly positive (Figure 1 and Table 4). This mutant, however, contains another mutation at position 120 leading to replacement of proline with serine. This mutation could have a significant effect on reactivity of some mAbs^[30,38] and has also been reported as a vaccine escape mutant with reduced binding affinity to anti-HBs Ab^[13]. To distinguish whether the P120S or S143L mutation is responsible for diminished reactivity of the 1056 mutant, we looked at binding patterns of M5 mutant. This mutant contains an S143L mutation together with 7 more mutations, mostly located within the first and second loops of “a” determinant, but not the P120S substitution. Reactivity of 3 of the 1056 non-reactive or low-reactive mAbs (37C5-S7, 35C6-S11 and 47G3-S15C) was restored, suggesting direct or indirect influence of this residue on their binding activity. Since the M5 mutant contains 8 substitutions, a definite conclusion cannot be drawn from this comparison. Ideally, a mutant with a single mutation at P120 is required to study the influence of this residue.

Altogether, from the reactivity patterns observed in

this study, our mAbs could be broadly classified into four distinct, but overlapping groups (Table 4). The first group includes 34D7-S3, 33D7-S4 and 38F3-S6 mAbs. Reactivity of these mAbs with the 1056 mutant distinguished this group of mAbs from other groups. None of these mAbs reacted with PA17D, M5 and BA3.2 mutants, carrying multiple mutations extending from residue 100 to 145. Lack of reactivity with BA3.2 (T123N/C124R) that has a cysteine to arginine substitution at position 124 could largely be attributed to low immunoreactivity or a low secretion rate of this mutant from transfected cells (Tables 3, 4). This mutation resulted in disruption of the disulphide bond of the first loop of “a” determinant with drastic conformational changes leading to retention of the molecule in transfected cells. Complete lack of secretion of this mutant by transfected COS7 cells has already been reported by two groups^[20,30] who demonstrated the presence of low levels of BA3.2 molecule in cell lysate, but not in the culture supernatant of transfected cells. In the present study, however, BA3.2 was readily detected in culture supernatant of the transfected cells by three diagnostic kits, as well as two of mAbs (37C5-S7 and 35C6-S11). Loss of reactivity of the first group of mAbs (34D7-S3, 33D7-S4 and 38F3-S6) with BA3.2 seems to be primarily affected by conformational changes due to disruption of the disulphide bond. Indeed, these mAbs recognize highly conformational overlapping epitopes, as judged from the Western blot (data not shown) and cross-inhibition results (Table 5).

The BA3.2 reactive mAbs (37C5-S7 and 35C6-S11) constitute the second group of mAbs. Reactivity of 37C5-S7 and, to a lesser extent, 35C6-S11 with BA3.2 could be due to the linear nature of the epitopes recognized by both mAbs. However, the reduced binding activity of these antibodies with BA3.2 seems to be affected by the T123N mutation. Furthermore, recognition of three other mutants, namely PA17D, M5 and BA2.4 having multiple mutations, by these two mAbs makes a distinction between this group of mAbs with other groups. A variety of mutations exist within the first and second loops of “a” determinant of these mutants, including residues 133, 134, 144 and particularly 145, which are critical for binding many of the mAbs^[31]. These mutations, however, do not significantly influence reactivity of 37C5-S7 and 35C6-S11 mAbs. Instead, a few mutations located outside these loops at positions 120 (1056), 123 (BA 3.2), 133 and 161 (SA 4) induced a significant reduction of reactivity of 37C5-S7 and 35C6-S11 mAbs with the corresponding mutants. The reduced reactivity observed for both mAbs with 1056 (P120S, S143 L) and SA 4 (M133 T/Y 161F) mutants does not seem to be related to replacements of 143 and 133 residues, because the same mutations are found in 91-4696 and SA7 mutants which strongly bind to both mAbs (Table 4). Therefore, the P120S, T123 N and M161T mutants are suspected to contribute to formation of the linear epitopes recognized by 37C5-S7 and 35C6-S11 mAbs. Amino acid 161 has been proposed to be located at the surface of HBsAg^[39] which could potentially influence antigenicity of the molecule. Such escape and natural mutants with altered antigenicity have already been reported^[40,41]. Deletion mutagenesis and phage display technology may help to

map these epitopes more precisely.

The third group of mAbs includes only one member, that is 47G3-S15C which cannot bind to either of four mutant proteins (1056, PA17D, BA3.2 and SA4), but reacts with M5 and BA2.4 mutants. The most influential mutations are thought to be P120S, T123N and Y161F similar to the pattern predicted for the second group of mAbs. However, unlike 37C5-S7 and 35C6-S11 mAbs, 47G3-S15C does not bind to the PA17D mutant. This molecule has been reported to carry a mutation at position 144 (D→E), as well as many other undisclosed residues^[30], making it difficult to assign the contributing residues. Involvement of D144E mutation seems to be unlikely, since this mAb reacts with the M5 mutant having multiple mutations at both sides of this amino acid, including P142S, S143L and G145R. This mAb may recognize a conformational epitope within the “a” determinant which is affected by mutations outside this region. Interestingly, mutations at residues close to the “a” determinant, particularly at positions 120 (P→Q or P→S) and 123 (T→N), were found to have a significant impact on the conformation and antigenicity of the “a” region epitopes leading to loss of reactivity of some “a” determinant-specific mAbs^[30,38].

The fourth group of mAbs is comprised of 35G9-S15A and 31D4-H12 antibodies. Both mAbs did not react with four mutants (1056, PA17D, M5 and BA3.2) and reacted weakly with two other mutants (BA2.4 and SA4). Although the epitopes recognized by 35G9-S15A and 31D4-H12 are structurally different (conformational and linear, respectively), they overlap to some extent (Table 5), suggesting their close spatial location on HBsAg. Considering the number of mutated residues in non-reactive and low-reactive mutants, assignment of the contributing amino acids and localization of the corresponding epitopes could not be predicted with certainty. Employment of mutants with overlapping mutations at one or two amino acids covering the entire major hydrophilic region and particularly the “a” determinant will help to map the epitopes and to determine the crucial amino acids involved in interaction with our anti-HBs mAbs.

In summary, our results could have important clinical implications for immunoscreening and diagnosis of HBV infection and design of new generation of recombinant HB vaccines. Although recent epidemiological survey failed to demonstrate close association between breakthrough infection and mutant HBV variants in vaccinated children in Taiwan^[26], incorporation of such mutants within the current standard recombinant HBs vaccines may later be considered as the new generation vaccines to prevent transmission of HBV mutants.

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Detection of *H pylori* infection by ELISA and Western blot techniques and evaluation of anti CagA seropositivity in adult Turkish dyspeptic patients

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Abstract

AIM: To detect *H pylori* infection and to evaluate the anti CagA seropositivity in adult Turkish dyspeptic patients.

METHODS: We evaluated anti-*H pylori* IgA, IgG and anti-CagA antibodies using commercial enzyme-linked immunoassay (ELISA) and Western blot in dyspeptic Turkish patients. *H pylori* status was determined by histology and rapid urease testing.

RESULTS: Fifty-six patients were entered. Forty-eight (85.7%) out of the 56 patients were positive for *H pylori*. *H pylori* IgG seropositivity was 82.1%, IgA seropositivity 48.2%. CagA ELISA showed that IgG was positive in 50% and IgA in 30.4% of those with *H pylori* infections. Western blot showed that IgG seropositivity was 80.4% and IgA seropositivity 33.9%. Western blot detected IgG antibodies with reactivity to CagA in 50%, VacA in 62.5%, UreB in 87.5%, UreA in 80.4%, and OMP in 57.1%. None of the tests had a sensitivity and specificity above 80%.

CONCLUSION: None of these commercial tests seems clinically useful for *H pylori* detection in adult dyspeptic patients, while Western blot can give seropositivity and determine anti-CagA, VacA virulence factor status of Turkish dyspeptic patients in the Izmir region.

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Key words: *H pylori*; Serum CagA; Enzyme-linked

INTRODUCTION

H pylori colonizes in the mucosa of the human stomach where it establishes a long-term infection associated with acute or chronic gastric inflammation, which may progress to peptic ulcer disease, atrophic gastritis with intestinal metaplasia, or gastric cancer. A variety of clinical outcomes of *H pylori* infection are associated both with host factors and with bacterial virulence factors^[1]. Several *H pylori* virulence genes have been identified, of these, oipA, vacA, cagA and babA appear to play a major role in pathogenicity. The cytotoxin-associated gene (CagA) is a marker for the cag pathogenicity island (PAI), a 40-kb genomic region^[1,2]. Most strains of *H pylori* from patients with peptic ulcer disease carry the CagA gene (CagA positive strains)^[1] and the presence of the CagA gene increases the risk of developing peptic ulceration, atrophic gastritis, and adenocarcinoma in the stomach. Consequently the discrimination of CagA positive and CagA negative *H pylori* strains might prove useful in predicting the chance for complications as well as for clinical and epidemiological studies of *H pylori* infection^[1,2].

H pylori cag PAI status is typically assessed by the immune response to the immuno-dominant CagA or by detection of the CagA gene. Available serological tools to characterize the infecting *H pylori* strains have been questioned because of their inadequate sensitivity and specificity^[3].

Current guidelines for the management of *H pylori* infection recommend eradication treatment without performing endoscopy in patients (< 45 years of age) with no alarm symptoms, thus making the availability of simple and reliable noninvasive tests important^[4]. Currently

available noninvasive tests for the diagnosis of *H pylori* infection include UBT, stool antigen test, and detection of anti-*H pylori* antibodies (e.g., serology).

This study compared five different diagnostic tests: rapid urease test, histology, anti-*H pylori* CagA Enzyme-linked immunoassay (ELISA) of IgA and IgG, anti-*H pylori* ELISA of IgA and IgG, Western blot of IgA and IgG including CagA and other antigens in untreated adult dyspeptic Turkish patients.

MATERIALS AND METHODS

Patients

The study population consisted of adult Turkish dyspeptic patients admitted to the Dokuz Eylül University Hospital, Gastroenterology Clinic. The patients were eligible if they had no *H pylori* eradication treatment in the previous 6 mo or did not receive antibiotics, H₂-receptor antagonists, sucralfate or omeprazole one month prior to examination, and had no previous history of gastric or duodenal malignancies. The patients with a history of coagulopathy or other disorders that were contraindications for endoscopy and/or biopsy sampling were excluded.

Endoscopy and biopsy sampling

Two antrum and two corpus biopsy specimens were taken from each patient undergoing upper endoscopy: one from the antrum and one from the corpus were used for the rapid urease test and the others 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. Hematoxylin and eosin, alcian blue and Giemsa stains were used for morphologic examination of *Helicobacter*-like organisms (HLO). The updated Sydney system was used for determining gastritis activity and grading bacterial density of *H pylori*. Gastritis activity was graded on a four-point scale as none (grade 0), mild (grade 1), moderate (grade 2), and severe (grade 3)^[5]. *H pylori* infection was defined as positivity of histopathology and rapid urease test. Histology was performed by a specialized pathologist. A patient was defined as *H pylori* negative when both histological examination and urease test were negative and as *H pylori* positive when both histological examination and urease test were positive.

Serological tests and sera

Sera were collected on the same day as the biopsies from patients undergoing endoscopy. Serum samples were aliquoted and stored at -20°C until used.

Enzyme-linked immunoassay

Anti-*H pylori* IgA and IgG Western blot, IgA and IgG ELISA, anti-CagA IgA and IgG ELISA (EUROIMMUN Medizinische Labordiagnostika, Lübeck, Germany) were used to detect the presence of *H pylori*-specific serum antibodies according to the manufacturer's instructions. The recommended cut-off values were used.

Immunoblot assay

Western blot (EUROIMMUN Medizinische Labordiagnostika, Lübeck, Germany) was used to detect the presence of *H pylori*-specific serum antibodies according to the manufacturer's instructions. Western blot test consisted of *H pylori* antigen extracts with the following molecular weights of the corresponding bands to these proteins which were 120 kDa (CagA); 95 kDa (VacA); 67 kDa (flagellar sheat protein, nonspecific); 66 kDa (UreB); 57 kDa (heat-shock protein homolog); 33 kDa, 30 kDa, 29 kDa (UreA); 26 kDa, 19 kDa and 17 kDa. Anti-*H pylori* IgG antibodies were positive determined by Western blot when the 120 kDa (CagA) band, as well as at least two distinctive antigen bands from species-specific and highly specific antigens with the molecular weights of 95 kDa (VacA), 33 kDa, 30 kDa, 29 kDa (UreA), 26 kDa, 19 kDa and 17 kDa were present. Faint bands or no band was regarded as negative.

Statistical analysis

The McNemar's χ^2 test was used. The sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV), the likelihood ratios (+, -) and diagnostic accuracies of the anti-*H pylori* CagA IgA and IgG ELISA, IgA and IgG ELISA were computed against the gold standards (SPSS, version 11.0 for Windows). The receiver operating characteristic (ROC) curve analysis was also done.

Ethics

The research protocol was approved by the Institutional Review Board and the Ethical Committee of the Dokuz Eylül University, Faculty of Medicine, Izmir, Turkey. All patients gave their written consent to participate in the study.

RESULTS

A total of 56 adult Turkish dyspeptic patients (19 males, 37 females; mean age, 46.41 \pm 13.12 years; age range, 21 to 78 years) were enrolled. *H pylori* infection was diagnosed in 48 (85.7%) patients by rapid urease test and histopathology. ELISA showed that *H pylori* seropositivity was 48.2% for IgA and 82.1% for IgG antibodies. CagA ELISA showed that IgA seropositivity was 30.4% and IgG seropositivity was 50% which was the same as the Western blot results. Western blot showed that IgA was positive in 33.9% and IgG in 80.4%. No significant statistical difference was found between CagA IgA and IgG antibodies by CagA ELISA but anti-*H pylori* IgG ELISA was significantly different with gold standards ($P < 0.05$) (Table 1, Table 2, Table 3).

Anti-*H pylori* Western blot of IgG antibodies also showed reactivity with p120 (CagA), p95 (VacA), p66 (UreB), p29 (UreA) and p19 (OMP) antigens: 28, 35, 49, 45 and 32 were positive, respectively (Table 4). A significant correlation with gastritis activity was also observed according to updated Sydney system ($P < 0.0001$). ROC analyses of all serological tests used in the study were also done and did not show any difference.

Table 1 Detection of *H pylori* status by Western blot, ELISA and CagA ELISA of IgA

Patients	Western blot <i>n</i> (%)			<i>H pylori</i> IgA ELISA <i>n</i> (%)		CagA ELISA <i>n</i> (%)		Total
	+	-	±	+	-	+	-	
<i>H pylori</i> (+) (<i>n</i> = 48)	17 (89.5)	24 (80.0)	7 (100)	26 (96.3)	22 (75.9)	16 (94.1)	32 (82.1)	48 (85.7)
<i>H pylori</i> (-) (<i>n</i> = 8)	2 (10.5)	6 (20.6)	0	1 (3.7)	7 (24.1)	1 (5.9)	7 (17.9)	8 (14.3)
Total	19	30	7	27	29	17	39	56

Table 2 Detection of *H pylori* status by Western blot, ELISA and CagA ELISA of IgG

Patients	Western blot <i>n</i> (%)			<i>H pylori</i> IgG ELISA <i>n</i> (%)		CagA ELISA <i>n</i> (%)		Total
	+	-	±	+	-	+	-	
<i>H pylori</i> (+) (<i>n</i> = 48)	40 (88.9)	1 (50.0)	7 (77.8)	42 (91.3)	6 (85.7)	26 (92.9)	22 (78.6)	48 (85.7)
<i>H pylori</i> (-) (<i>n</i> = 8)	5 (11.1)	1 (50.0)	2 (22.2)	4 (8.7)	4 (57.1)	2 (7.1)	6 (21.4)	8 (14.3)
Total	45	2	9	46	10	28	28	56

Table 3 Results of anti-*H pylori* ELISA and CagA ELISA of IgA, IgG in dyspeptic patients

	Sensitivity %	Specificity %	PPV %	NPV %	LR +	LR -	Diagnostic accuracy %
ELISA IgA	54.2	87.5	96.3	24.1	0.62	4.33	58.9
ELISA IgG	87.5	50	91.3	40	1.75	1.75	82.1
CagA ELISA IgA	33.3	87.5	94.1	18	0.38	2.67	41.1
CagA ELISA IgG	54.2	75	92.9	21.4	0.72	2.17	57.1

PPV: Positive predictive value; NPV: Negative predictive value; LR: Likelihood ratio.

Table 4 Anti-*H pylori* IgG Western blot results of the distribution of different antigens

Antigens	Positive patients (<i>n</i> = 56)	%
p120 (CagA)	28	50
p95 (VacA)	35	62.5
p75	18	32.1
p67 (FSH)	23	41.1
p66 (UreB)	49	87.5
p57 (HSP homolog)	37	66.1
p54 (Flagellin)	24	42.9
p50	24	42.9
p41	28	50
p33	13	23.2
p30	24	42.9
p29 (UreA)	45	80.4
p26	23	41.1
p19 (OMP)	32	57.1
p17	20	35.7

DISCUSSION

H pylori infection can be diagnosed by a variety of invasive and non-invasive tests. Serology can be performed on non-invasively collected clinical samples. Serological detection of infection with a CagA containing strain of *H pylori* by anti-CagA ELISA and Western blot of CagA is the only noninvasive diagnostic test at present available for assessing strain virulence potential and possible disease risk. The reliability of CagA serology as a predictive test for determining the CagA genotype of the infecting strain is important because various serological assays are now available^[2].

Infection with *H pylori* evokes both local and systemic antibody responses^[6]. CagA is the important pathologic marker with a high immunogenic response^[6,7]. In Europe, CagA-positive *H pylori* has been reported to account for 60% to 70% of *H pylori* strains^[2,8], while reports from East Asian countries have shown that more than 90% of *H pylori* strains are CagA positive irrespective of the disease presentation^[8].

Noninvasive tests are becoming more and more important in the clinical management of dyspeptic patients, followed by the treatment of *H pylori* in primary care according to “the Maastricht 2-2000 European Consensus report^[4]”. A “test and treat” approach is recommended in adult patients under the age of 45-55 years with no alarm symptoms. But choosing the ‘right’ noninvasive test is not easy. Serological tests have limitations in detecting the exposure to *H pylori*. A meta-analysis of 21 studies with commercially available ELISA serology kits has reported an overall sensitivity and specificity of 85% and 79%, respectively. Thus, the accuracy of these tests is no longer adequate to justify their use on clinical or economic grounds. However, new serological kits are available to detect antibodies to CagA or VacA by ELISA and Western blot, and can achieve rather good sensitivity and specificity^[9,10]. Because serological tests for *H pylori* vary

in different populations, largely due to the *H pylori* strain heterogeneity and variations in antigenic preparations, their accuracy must be confirmed in the target populations^[11,12]. Unfortunately, none of these new tests has a sensitivity and specificity above 80% and therefore they should be used with care for *H pylori* detection in Turkish dyspeptic patients in our region.

Serin *et al*^[13] compared the frequencies of serum positive CagA in patients from two separate regions of Turkey and found that the rate of CagA serum prevalence is high (97.2%) in patients with non-ulcer dyspepsia but similar in *H pylori*-positive patients. They also observed similar frequencies of CagA (+) *H pylori* strains in dyspeptic patients irrespective of ulcer status, suggesting that factors other than CagA can contribute to severe gastrointestinal pathology in patients with *H pylori*. We found that 50% of patients with *H pylori* infection had CagA positive serology which is below the percentage expected, suggesting that the test is not accurate.

A set of serological tests may give more accurate determinations of *H pylori* infection than one test detecting specific antibody or bacterial antigen. In this study it seemed that there was a good correlation with Western blot and ELISA test results compared to the gold standard methods. *H pylori* seropositivity was 82.1% by ELISA IgG antibodies and 80.4% by Western blot IgG antibodies. We suggest that ELISA, CagA ELISA and Western blot techniques should be used together where both the incidence of *H pylori* and the treatment failure rates are very high. We should emphasize more on anti-CagA status determination because anti-CagA positive patients are substantially associated with atrophic gastritis, persistent active inflammation and atrophic gastritis.

In conclusion, anti-*H pylori* IgA testing seems clinically useless and IgG testing is not clinically possible because the tests are based on antigens from European strains. Since Izmir is a cosmopolitan area under the influence of both Asian and Western countries, the common strains may differ significantly from those in Europe.

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Dose-related effects of dexamethasone on liver damage due to bile duct ligation in rats

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significant reduction of liver damage without increased side effects, while high dose is associated not with lower fibrosis but with increased side effects.

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Key words: Bile duct ligation; Hepatic fibrosis; Dexamethasone

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Abstract

AIM: To evaluate the effects of dexamethasone on liver damage in rats with bile duct ligation.

METHODS: A total of 40 male Sprague-Dawley rats, weighing 165-205 g, were used in this study. Group 1 (sham-control, $n = 10$) rats underwent laparotomy alone and the bile duct was just dissected from the surrounding tissue. Group 2 rats (untreated, $n = 10$) were subjected to bile duct ligation (BDL) and no drug was applied. Group 3 rats (low-dose dexamethasone, $n = 10$) received a daily dose of dexamethasone by orogastric tube for 14 d after BDL. Group 4 rats (high-dose dexamethasone, $n = 10$) received a daily dose of dexamethasone by orogastric tube for 14 d after BDL. At the end of the two-week period, biochemical and histological evaluations were processed.

RESULTS: The mean serum bilirubin and liver enzyme levels significantly decreased, and superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) values were significantly increased in low-dose dexamethasone and high-dose dexamethasone groups when compared to the untreated group. The histopathological score was significantly less in the low-dose and high-dose dexamethasone groups compared to the untreated rats. In the low-dose dexamethasone group, moderate liver damage was seen, while mild liver damage was observed in the high-dose dexamethasone group.

CONCLUSION: Corticosteroids reduced liver damage produced by bile duct obstruction. However, the histopathological score was not significantly lower in the high-dose corticosteroid group as compared to the low-dose group. Thus, low-dose corticosteroid provides a

INTRODUCTION

Obstruction of bile flow through the extrahepatic biliary system results in development of oxidant injury, hepatic fibrosis, biliary cirrhosis, and portal hypertension^[1,2]. Patients with obstructive jaundice are at significant risk for severe complications, particularly sepsis^[3,4]. Although preoperative percutaneous biliary drainage has been attempted to reduce perioperative complications, it failed to demonstrate a significant benefit despite successful reduction in serum bilirubin levels^[5]. Preventing or minimizing the deleterious effects of bile acids might represent a potential therapeutic target for patients with obstructive jaundice. However, effective pharmacological therapy in chronic liver diseases is not yet available.

Since the inflammatory process is important in the development of liver injury following biliary obstruction, the purpose of this study was, therefore, to evaluate the effects of dose-related dexamethasone (dexa) on liver damage caused by bile duct ligation in rats.

MATERIALS AND METHODS

Forty male Sprague-Dawley rats (Dicle University Research Center), weighing 165-205 g, were used in the study. All of the experimental protocols were performed according to the guidelines for the Ethical Treatment of Experimentation Animals. The animals were divided into 4 groups, each group containing 10 animals. Each rat was anesthetized with ketamine (50 mg/kg) and xylazine (4 mg/kg). The rats were subjected to either bile duct ligation (BDL) or sham operation using aseptic

techniques, as previously described by Criado *et al*^[6]. Group 1 rats (sham-control) underwent laparotomy alone and the bile duct was dissected from surrounding tissue. Group 2 rats (untreated) were subjected to bile duct ligation alone and received 1 mL of saline by orogastric tube. Group 3 rats (low-dose dexamethasone) received a daily dose of dexamethasone (0.125 mg/kg per day) by orogastric tube for 14 d after BDL. Group 4 rats (high-dose dexamethasone) received a daily dose of dexamethasone (0.400 mg/kg per day) by orogastric tube for 14 d after BDL. The rats were housed in standard cages in a room at 12 h:12 h light-dark condition, temperature (20°C) and humidity (60%), and maintained on a standard rat pellet diet. At the end of the two-week period, all animals were anesthetized with 100 mg/kg inactin ip, placed on a thermoregulated table, and a short segment of polyethylene (PE)-240 catheter was inserted into the trachea to assist the spontaneous respiration. After opening the abdomen through a midline incision, the abdominal aorta was punctured and 5 mL of blood was taken into heparinized tubes. Plasma was separated by centrifugation for biochemical studies, and the activities of alanine aminotransferase (ALT) (IU/L), aspartate aminotransferase (AST) (IU/L), alkaline phosphatase (ALP) (IU/L), γ -glutamyltranspeptidase (GGT) (IU/L) and the concentrations of total bilirubin (TB) (mg/dL) in plasma were determined by standard auto-analyzer methods on an Abbot Aeroset (USA). Just before the rats were sacrificed, the livers were extracted for histopathological evaluation. During this period of surgical preparation, the rats in all groups received 1% of their body weight of Ringer's lactate solution.

Histopathological examination

The extracted liver was divided into two pieces in each rat. One of the pieces was immediately placed into 100 g/L formaldehyde solution overnight, embedded in paraffin, and cut into 5-mm thick sections; stained either with hematoxylin-eosin (HE) or Masson's trichrome for light microscopic analysis. Histopathological scoring of groups for degree of fibrosis (ductular proliferation, focal ductular cholestasis, portal tract expansion, mixed inflammation, necrosis, and fibrosis) was scored as: 0 = absent; 1 = slight; 2 = moderate; and 3 = severe^[7]. Histopathological evaluation was performed twice in four sections per slide from all animals in each group. In addition, the number of infiltrating neutrophils per portal tract was assessed by counting neutrophils manually at a 400 \times magnification (Olympus Eyepiece Micrometer[®]) in 10 portal tracts per slide ($n = 10$ in each group).

The other piece was washed in ice-cold saline and homogenized in 0.1 mol/L Tris-HCl buffer (pH 7.4). The homogenate was then centrifuged and the supernatant obtained was used for the assay of various enzymes. Superoxide dismutase (SOD) was assayed as previously described by Misra and Fridovich^[8]. Based on the inhibition of epinephrine auto-oxidation by the enzyme, catalase (CAT) activity was measured by following decomposition of H₂O₂ according to the method described by Beers and Sizer^[9]. Glutathione peroxidase (GSH-Px) was assayed using H₂O₂ as the substrate as previously described^[10].

Table 1 Comparative biochemical measurements at the two week of the study (mean \pm SD)

Groups	Biochemical parameters				
	ALP (IU/L)	GGT (IU/L)	TB (mg/dL)	AST (IU/L)	ALT (IU/L)
Sham-control	177 \pm 20	1.9 \pm 0.3	0.3 \pm 0.2	117 \pm 3.3	55 \pm 10
Untreated	386 \pm 44 ^a	49 \pm 10 ^a	11 \pm 1.3 ^a	1378 \pm 256 ^a	258 \pm 62 ^a
Low-dose dexamethasone	297 \pm 78 ^c	20 \pm 2.3 ^c	8 \pm 0.8 ^c	1037 \pm 215 ^c	198 \pm 39 ^c
High-dose dexamethasone	300 \pm 60 ^e	19 \pm 2 ^e	8 \pm 1.1 ^e	963 \pm 139 ^e	150 \pm 43 ^e

ALP: Alkaline phosphatase; GGT: γ -glutamyltranspeptidase; TB: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase. ^a $P < 0.05$ vs sham-control; ^c $P < 0.05$ vs untreated; ^e $P < 0.05$ vs untreated.

Table 2 Comparative SOD, CAT and GSH-Px measurements at the two week of the study (mean \pm SD)

Groups	SOD (IU/L)	CAT (IU/L)	GSH-Px (mg/dL)
Sham-control	16 \pm 2.5	5.1 \pm 0.9	430 \pm 31
Untreated	6.9 \pm 0.9 ^a	0.8 \pm 0.1 ^a	142 \pm 13 ^a
Low-dose dexamethasone	10.6 \pm 1.2 ^c	2.7 \pm 0.6 ^c	254 \pm 15 ^c
High-dose dexamethasone	10.7 \pm 0.9 ^e	2.6 \pm 0.6 ^e	255 \pm 19 ^e

SOD: Superoxide dismutase; CAT: Catalase; GSH-Px: Glutathione peroxidase. ^a $P < 0.05$ vs sham-control; ^c $P < 0.05$ vs untreated; ^e $P < 0.05$ vs untreated.

Statistical analysis

Data were entered and analyzed on an IBM compatible personal computer using SPSS version 9.0. All values were expressed as mean \pm SD. The significance of the data obtained was evaluated by using analysis of variance (ANOVA). Differences between means were analyzed by using the post-ANOVA (Tukey's b) test. $P < 0.05$ was considered statistically significant.

RESULTS

The mean weights of the animals from the four groups before and after BDL were, respectively, as follows: 192 \pm 15 g vs 196 \pm 9 g, 204 \pm 12 g vs 166 \pm 3 g, 203 \pm 12 g vs 167 \pm 9 g, and 190 \pm 9 g vs 119 \pm 11 g. These data showed that the mean weights of the three study groups decreased significantly compared to the control group after BDL ($P < 0.0001$ for all). In addition, the mean weight of group 4 rats decreased significantly compared to groups 2 and 3 ($P < 0.0001$ for all).

The values of biochemical measurements for the different groups are shown in Table 1. Serum levels of ALP, GGT, TB, AST and ALT were significantly increased in the untreated, low-dose dexamethasone and high-dose dexamethasone groups in comparison with the sham-control group (all $P < 0.05$). ALP, GGT, total bilirubin, AST and ALT values were decreased in the low-dose dexamethasone group and high-dose dexamethasone group when compared to the untreated group (all $P < 0.05$). However, no significant difference was found between the low-dose dexamethasone and high-dose dexamethasone groups.

The values of SOD, CAT and GSH-Px measurements for the different groups are shown in Table 2. Mean values were significantly decreased in the untreated, low-dose

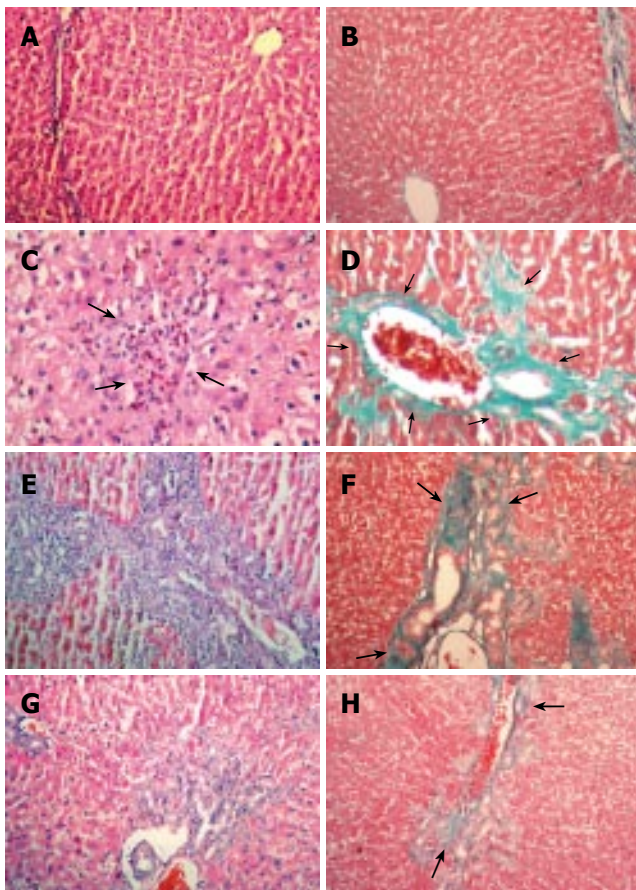


Figure 1 Morphological changes in the livers of different group rats (HE $\times 200$; Masson's trichrome $\times 200$). No morphological damage was observed in any of the rats in the sham-control group (A and B). In the untreated group, proliferation of portal and periportal biliary ductules with disorganization of the hepatocytes plates, dilated portal spaces and areas of polymorphonuclear leukocyte infiltrate, hepatocytes necrosis and fibrosis were observed (C and D). Moderate hepatocytes necrosis and fibrosis were present in the low-dose dexamethasone group (E and F). The high-dose dexamethasone group showed a remarkably less necrosis and fibrosis (G and H). A, C, E and G are stained with HE; B, D, F and H are stained with Masson's trichrome.

dexamethasone and high-dose dexamethasone groups in comparison with the sham-control group (all $P < 0.05$). However, these values were significantly increased in low-dose and high-dose dexamethasone groups when compared to the untreated group (all $P < 0.05$).

The mean count of polymorphonuclear leukocytes (PNL) in the control group was less than one in portal spaces of hepatic parenchyma, whereas it was 15 ± 3 , 4.6 ± 0.9 and 3.4 ± 0.8 in the untreated, low-dose dexamethasone and high-dose dexamethasone groups, respectively. The number of PNL was significantly decreased in low-dose and high-dose dexamethasone groups when compared to the untreated group ($P < 0.0001$). However, no significant difference was found between the high-dose dexamethasone and sham-control group.

No morphological damage was observed in any of the rats in the sham-control group (Figure 1A, 1B). In the untreated group, severe damage (dilated central veins, proliferation of portal and periportal biliary ductules with disorganization of the hepatocyte plates, dilated portal spaces and hepatocyte necrosis) was observed (Figure 1C, 1D). In the low-dose and high-dose dexamethasone groups, moderate damage (dilated central veins and minimal disorganization of the hepatocyte plates, and hepatocyte necrosis) was

observed (Figure 1F). Mild damage (less necrosis and less fibrosis) was observed in the high-dose dexamethasone group (Figure 1G, 1H).

The histopathological scores found in the sham-control, untreated, low-dose dexamethasone and high-dose dexamethasone groups were 0.1 ± 0.3 , 2.6 ± 0.5 , 1.9 ± 0.7 and 1.8 ± 0.6 , respectively. The histopathological score tended to be more in the untreated, low-dose dexamethasone and high-dose dexamethasone groups as compared with the sham-control groups (all $P < 0.0001$). However, the histopathological score was significantly less in the low-dose and high-dose dexamethasone groups compared to the untreated rats ($P < 0.05$, $P < 0.02$, respectively). Moreover, no significant difference was observed in the histopathological score between the low-dose dexamethasone and high-dose dexamethasone groups.

DISCUSSION

Bile duct ligation (BDL) in rats induces portal fibrosis, which begins with an early proliferation of biliary duct epithelial cells and portal periductular fibroblasts^[11]. In the early phases, there is neither substantial hepatocyte necrosis, nor fibrotic reaction in the centrilobular region^[12]. Later, fibrosis distributes more widely and alters intrahepatic vascular resistances and blood flow, leading to the establishment of portal hypertension^[13]. In this experimental study, the rats subjected to BDL for two weeks showed changes in plasma levels of bilirubin, AST, GGT and ALP, thereby indicating the presence of cholestasis and diffuse hepatic injury. These observations are in agreement with those of several authors^[14,15]. After BDL, we observed a significant decrease in the mean weights of rats, including group 4. It has been demonstrated in previous studies that the administration of dexamethasone was ineffective in increasing the liver weight in advanced liver fibrosis in rats or in improving body weight or body weight gain^[16]. In fact, the body weight (or body weight gain) was further decreased by dexamethasone treatment in rats with liver fibrosis^[17], which is in agreement with our study.

BDL affects the balance between antioxidant and prooxidant activities and increases the prooxidant activity. It increases production of free radicals^[18] and decreases free radical scavengers (GSH-Px, SOD and CAT). High level of free radicals induces lypoperoxidation^[19] and alteration of fluidity and functionality of cell membranes^[20]. It has been suggested that the increase of intrahepatocyte concentration of biliary acids induces mitochondrial toxicity in chronic cholestasis^[24]. Furthermore, increased lipid peroxidation in the BDL model has been described^[21]. A significant correlation between TB levels and lipid peroxidation has also been described in hepatic toxicity or biliary tract diseases^[22]. In addition, nitric oxide (NO) has been shown to be related to the development of hepatic fibrosis. The hepatocytes are mainly responsible for the production of NO radicals^[23]. Dexamethasone can either inhibit the release of inflammatory mediators and consequently the formation of NO radicals or block the up-regulation of inducible NO synthetase^[24]. Also, previous studies have indicated a dual role for dexamethasone; besides blocking

the release of pro-inflammatory cytokines, it also reduces the release of anti-fibrotic mediators by Kupffer cells and sinusoidal endothelial cells in the liver^[25,26]. In our study, dexamethasone protected the levels of such antioxidant enzymes as SOD, CAT and GSH-Px in both doses.

The main liver injury following biliary obstruction is periductal inflammation, bile duct proliferation and portal fibrosis. Prominent among the inflammatory cells that invade obstructed livers are neutrophils; it has been evidenced by several experimental studies that neutrophils infiltrate the liver within 3 h of BDL^[27], and remain there for days to weeks as fibrosis progresses^[27-29]. In addition, dexamethasone was found to prevent infiltration of inflammatory cells, PMNs and monocytes *in vivo*^[16,30]. A connection between neutrophils and liver fibrosis was first suggested by Parola *et al*^[27]. They quantitated hepatic neutrophils in an experimental model of bile duct obstruction and found that the number of infiltrating cells correlated directly with the degree of liver fibrosis. In the present study, we found that the number of PNL decreased in the low-dose and high-dose dexamethasone groups, which is in accordance with previous studies.

Glucocorticoids are used as anti-inflammatory and immunomodulatory agents in a wide variety of diseases^[31]. Their physiological effects may be accomplished largely by modulating the expression of many cytokine genes, such as IL-1^[32], IL-2^[33], TNF- α ^[34], interferon- β ^[35], interferon- γ ^[33], and monocyte chemotactic and activating factor^[36]. Dexamethasone is also a phospholipase A2 inhibitor and has been shown to inhibit endothelin-1-stimulated arachidonic acid production^[37]. Dexamethasone also blocks conversion of arachidonic acid by cyclooxygenase into prostaglandins and suppresses the production of various inflammatory mediators^[38]. Although dexamethasone enhances hepatocyte viability and improves the expression of liver-specific genes in liver diseases, there is controversy over the beneficial effects of dexamethasone for liver fibrosis treatment. Dexamethasone has been used as an anti-inflammatory drug in the treatment of chronic active hepatitis for the prevention of liver fibrosis^[39]. Glucocorticoids suppress collagen synthesis and gene expression by fibroblasts. Nevertheless, glucocorticoids do not have an inhibitory effect on extracellular matrix synthesis by hepatic stellate cells^[16]. In our study, dexamethasone, particularly in high dose, decreased proliferation and infiltration of inflammatory cells in portal and periportal biliary ducts. The results of previous studies provide evidence that dexamethasone fails to therapeutically improve liver function during fibrosis. Thus, the beneficial effect of dexamethasone in ascites formation may be attributed to its anti-inflammatory effect and to a decrease in the production of vasoactive substances from inflammatory cells, but not to improve liver functions and decrease fiber accumulation^[16,40]. In our study, however, ALP, GGT, total bilirubin, AST and ALT values were decreased in the low-dose dexamethasone and high-dose dexamethasone groups compared to the untreated group. Although dexamethasone is widely used in the management of inflammatory diseases, it may cause such serious side-effects as immunosuppression and growth retardation, especially when used at high doses. In the present study,

parallel to the literature, dexamethasone caused growth retardation when used at high doses.

In conclusion, using corticosteroids reduced liver damage produced by bile duct obstruction. The finding that no significantly lower histopathological score in the high-dose dexamethasone group compared to the low-dose group suggests that high-dose dexamethasone does not provide any additional protective effect on liver damage. Moreover, high-dose dexamethasone causes obvious growth retardation. Thus, low-dose corticosteroid provides a significant reduction in liver damage without increased side effects, while high dose is associated not with lower fibrosis but with increased side effects.

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RAPID COMMUNICATION

Factors associated with *H pylori* epidemiology in symptomatic children in Buenos Aires, Argentina

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in this population (mean age 9.97 ± 3.1 years). The factors associated with *H pylori* positivity were number of siblings ($P < 0.001$), presence of pet cats ($P = 0.03$) and birds ($P = 0.04$) in the household, and antecedents of gastritis among family members ($P = 0.01$). After multivariate analysis, number of siblings [Odds ratio (OR) = 1.39; 95% CI, 1.20-1.61] and contact with pet cats (OR = 1.76; 95% CI, 1.00-3.09) remained as variables associated with *H pylori* infection.

CONCLUSION: The prevalence of *H pylori* infection in children with upper gastrointestinal symptoms in Argentina was similar to that reported in developed countries. Children from families with a higher crowding index and presence of pet cats have a higher risk of being colonized with *H pylori*.

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Key words: *H pylori*; Children; Epidemiology; Urea breath test; Prevalence

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Abstract

AIM: To determine prevalence of *H pylori* infection in symptomatic children in Buenos Aires, Argentina, and to investigate factors associated with *H pylori* positivity.

METHODS: A total of 395 children with upper gastrointestinal symptoms referred to the Gastroenterology Unit of the Children Hospital "Sor Maria Ludovica" were evaluated for the presence of *H pylori* by the ^{13}C -Urea Breath Test (^{13}C -UBT). A questionnaire was applied to the recruited population.

RESULTS: Prevalence of *H pylori* infection was 40.0%

INTRODUCTION

H pylori bacterium is now recognized as a major etiologic factor in the development of chronic superficial gastritis and peptic ulcer disease in adults and children^[1]. Because its association with gastric cancer *H pylori* was classified in 1994 as a group 1 carcinogen by the International Agency for Research on Cancer^[2]. *H pylori* acquisition occurs predominantly during early childhood, and its incidence and prevalence is higher in developing than in developed countries^[3-5]. Several risk factors have been associated with acquisition and transmission of *H pylori* infection, those factors are mainly correlated with poor sanitary conditions and low socioeconomic status^[5-7].

Controversial results have been found in establishing the role of *H pylori* as the etiology for the presence of specific symptoms in children such as recurrent abdominal pain (RAP)^[8,9]. Specific symptoms suggestive of acute *H pylori* infection are vague, inconsistent, and similar to several other more common childhood disorders, manifesting as recurrent abdominal pain, dyspepsia or epigastric pain^[10].

The aims of our study were to determine prevalence of *H pylori* infection in symptomatic children in Buenos Aires, Argentina, and to investigate risk factors associated with *H pylori* positivity.

MATERIALS AND METHODS

Subjects

The study was performed in 395 children with age ranging from 2 to 17 years (mean age 9.97 ± 3.1 years), who were referred to the Gastroenterology Unit of the Children Hospital "Superiora Sor Maria Ludovica" for upper gastrointestinal symptoms evaluation (gastroesophageal reflux, esophagitis symptoms, ulcerous syndrome, abdominal pain, and upper digestive haemorrhage). The Hospital is a tertiary level health care referral institution with the highest clinical complexity for attending children in the Province of Buenos Aires. The Gastroenterology Unit receives a monthly average of 600 patients who are referred from other services within the same hospital and from primary health care units located in the Province of Buenos Aires. Parents or grandparents identified as the responsible adults of the children, were instructed to carefully read the protocol information and to sign a written consent form according to the Helsinki declaration. Children with a signed consent were included in the study. Participation consisted in the diagnosis of *H pylori* infection by means of the ¹³C-Urea Breath Test (¹³C-UBT) and the completion of a questionnaire for epidemiological purposes.

¹³C-urea breath test (¹³C-UBT)

Children were instructed to fast for at least 6 h before the diagnostic test was performed. ¹³C-UBT consisted of the following: two samples of exhaled air were taken previous to the ingestion of the labeled solution to determine basal ¹³C/¹²C ratios. Then, 150 mL of reconstituted powdered non-fatty milk containing 50 mg of ¹³C-urea (Cambridge Isotope Laboratories Inc., Massachusetts, USA) were taken by each patient. Breath samples were collected at 30 and 45 min after the ingestion of the labeled solution in hermetically sealed containers (Labco limited, United Kingdom). Each sample of exhaled air was measured in a mass spectrometer coupled to a gas chromatographer (FinniganMAT GmbH, ThermoQuest Corp., Bremen, Germany). A change of > 3.5% in the delta over baseline (DOB) values was considered positive. The ¹³C-UBT is a highly accurate diagnostic test, with values of sensitivity and specificity over 95%^[11].

Epidemiological questionnaire

Parents or grandparents of the participant children were

instructed to complete a questionnaire for epidemiological purposes. The questionnaire was focused on variables that might affect the risk for *H pylori* positivity. The evaluated variables were demographic data, family crowding (number of siblings, rooms in the house), socioeconomic status and sanitary standards [type of house (masonry, wooden, rustic), type of flooring (wooden, cement, soil), type of toilet (sewer, septic tank, pit latrine), source of water (well-shaft treated, well-shaft not treated, treated system)], presence of pets in the house, food intake [raw food (uncooked meat, chicken or fish) *vs* cooked food], drinking of beverages shared from the same container (with a special focus on the consumption of "mate", a traditional argentine green herbs infusion), habit of chewing the nails, and history of digestive diseases among family members (gastritis, gastric and duodenal ulcers, or gastric cancer).

Statistical analysis

The Fisher Exact test was used to analyze dependency between *H pylori* positivity and other categorical variables. The Chi squared test was applied to variables with more than two categories. To analyze if variances of quantitative variables were homogeneous for both *H pylori* positive and negative groups, the Levene test was applied. Student's *t* test was used when it was proven that variances were homogeneous, if not, the non-parametric Mann-Whitney test was applied. A binary logistic regression equation was used to estimate the impact of different characteristics as predictive variables for *H pylori* status, by the Forward Stepwise (Likelihood Ratio) method. The results of logistic regression included odds ratios (OR) as well as 95% confidence intervals (CI) for each of the variables. Significance levels were set at alpha less than 0.05. The SPSS 10.0 statistical program (SPSS, Chicago, IL) was used to perform all the statistical analyses presented in this article.

RESULTS

All the 395 participating children were tested for *H pylori* infection by means of the ¹³C-UBT. A total of 158 patients were found to be *H pylori* positive. Prevalence of *H pylori* infection in this symptomatic population was 40.0% (95% CI, 35.2-44.8). Among the 395 enrolled children, 332 (84.1%) completed the epidemiological questionnaire. *H pylori* prevalence was similar between the studied population and those excluded from the analysis because of the lack of the questionnaire. Prevalence of *H pylori* infection among different age groups was as follows: 2-5 years (*n* = 29), 34.5%; 6-7 years (*n* = 47), 48.9%; 8-9 years (*n* = 59), 37.3%; 10-11 years (*n* = 75), 45.3%; 12-13 years (*n* = 73), 37.0%; 14-17 years (*n* = 44), 47.7%. Table 1 summarizes the demographics of the population included in the study. No significant differences were found among age (*P* > 0.70), gender (*P* > 0.60), ethnic group (*P* > 0.15), educational level (*P* > 0.50) and place of residence (inner city *vs* suburban areas) (*P* > 0.40), between *H pylori* positive and negative patients.

The most relevant factors evaluated to influence the prevalence of *H pylori* infection are shown in Table 2. As

Table 1 Demographical data of the children included in the study

	<i>H pylori</i> (+) n (%)	<i>H pylori</i> (-) n (%)	<i>P</i>
<i>n</i>	140	192	
Age (yr) (mean ± SD)	9.89 ± 3.16	10.02 ± 2.96	0.708
Gender			
Female	77 (54.9)	110 (57.4)	
Male	63 (45.1)	82 (42.6)	0.660
Ethnic group			
Caucasian	130 (93.1)	170 (88.7)	
Asian	0 (0.0)	4 (2.0)	
American Indian	10 (6.9)	18 (9.3)	0.156
Educational level			
Kindergarten	14 (9.8)	24 (12.4)	
BGE ¹	116 (83.2)	150 (78.4)	
Polimodal ²	10 (7.0)	18 (9.2)	0.564
Place of residence			
BA city ³	6 (4.2)	10 (5.2)	
Great BA ⁴	9 (6.3)	7 (3.6)	
BA province ⁵	125 (89.5)	175 (91.2)	0.491

¹ Basic general education (for children from 6 to 15 years old); ² For children from 15 to 18 years old; ³Buenos Aires city (inner city); ⁴Great Buenos Aires (suburban areas); ⁵Buenos Aires province (inner city).

an indicator of domestic crowding, “number of siblings” was significantly associated with *H pylori* positivity ($P < 0.001$). None of the variables depicting socioeconomic status and sanitary standards were correlated to the infection. On the other hand, we found a significant correlation between having contact with cats and birds, and being positive for the infection ($P = 0.02$ and $P = 0.04$ respectively). Neither the ingestion of raw or cooked food nor drinking of “mate” or other shared drinks were significantly linked to *H pylori*. The habit of chewing nails was also not correlated to the infection. We found a significant correlation between history of gastritis in family members and a positive *H pylori* result in the studied child (index case) ($P = 0.01$).

To estimate the impact of different characteristics as predictive variables for *H pylori* status, the binary logistic regression equation was used by the Forward Stepwise (Likelihood Ratio) method. Under this analysis, predictive variables for *H pylori* positivity were “contact with pet cats” (OR = 1.76; 95% CI, 1.00-3.09) and “number of siblings” (OR = 1.39; 95% CI, 1.20-1.61).

DISCUSSION

Prevalence of *H pylori* infection has been reported to be higher both in children and adults from developing countries than from developed ones^[10,12,13]. In Argentina, *H pylori* prevalence has been evaluated in the asymptomatic population in two different studies that included both adults and children^[14,15]. Mean age of the children in those previous studies was 7.9 ± 4.6 years^[14] and 7.8 ± 5.5 years^[15]. The prevalence of *H pylori* in asymptomatic children was 15.7% in both studies. In the present study we evaluated slightly older children with gastrointestinal symptoms (mean age 9.97 ± 3.1 years), and we found a

Table 2 Potential factors associated with *H pylori* positivity

	<i>H pylori</i> (+) n (%)	<i>H pylori</i> (-) n (%)	<i>P</i>
Domestic crowding			
Siblings			
0	3 (2.1)	10 (5.2)	
1	38 (27.1)	68 (35.4)	
2	23 (16.4)	58 (30.2)	
3	29 (20.7)	18 (9.4)	< 0.001 ^b
4	15 (10.8)	16 (8.3)	
5	9 (6.4)	10 (5.2)	
> 5	23 (16.5)	12 (6.3)	
Rooms in the house			
1	10 (7.1)	14 (7.3)	
2	61 (43.6)	83 (43.2)	
3	44 (31.4)	57 (29.7)	> 0.700
4	12 (8.6)	14 (7.3)	
5	13 (9.3)	24 (12.5)	
Contact with pets			
No	21 (14.7)	32 (16.6)	
Yes	119 (85.3)	160 (83.4)	0.407
Dog	101 (91.8)	132 (91.7)	0.577
Cat	46 (41.8)	42 (29.2)	0.025 ^a
Hamster	11 (10.0)	9 (6.3)	0.193
Reptile	6 (5.5)	7 (4.9)	0.518
Bird	20 (18.4)	14 (9.7)	0.036 ^a
History of digestive diseases among family members			
No	58 (41.4)	93 (48.5)	
Yes	82 (58.6)	99 (51.5)	0.105
GU ¹ or DU ²	24 (16.4)	30 (15.2)	0.429
Gastritis	74 (50.7)	75 (37.9)	0.012 ^a
GC ³	16 (11.2)	31 (15.8)	0.145

¹ Gastric Ulcer; ² Duodenal Ulcer; ³ Gastric Cancer; ^aSignificantly different ($P < 0.05$); ^bSignificantly different ($P < 0.001$).

40.0% prevalence of *H pylori* infection. The differences in prevalence rates found in asymptomatic and symptomatic children from our country, are consistent with one multicenter study representing various parts of the United States of America, in which the seropositivity rate was significantly higher in symptomatic (22.3%) than in asymptomatic children (14.1%)^[6]. In a study from the Czech Republic^[16], it was also reported a higher prevalence of *H pylori* infection in symptomatic children (33%) when compared with asymptomatic controls (7.5%). These findings suggest that the presence of gastrointestinal complaints may be associated with *H pylori*. Moreover, the lack of association between age and *H pylori* prevalence observed in this study could also be explained by the inclusion of symptomatic children only. Nevertheless, a causal relationship between *H pylori* infection and recurrent abdominal pain in children is still not proven^[8]. Although Argentina is considered a developing country, results obtained in the present study (40% *H pylori* prevalence in symptomatic children) versus 15% in asymptomatic children reported previously^[14,15] are interestingly similar to the ones obtained in developed countries such as the US.

According to the National Institute of Statistics and

Census of Argentina, INDEC, there is a 44.4 % poverty rate among the population living in Great Buenos Aires metropolitan area. A possible explanation that our studied population of children had good socioeconomic status and high sanitary standards is that more than 95% of the studied children were from inner city and they might mainly represent middle class. This phenomenon may be explained by the following reasons: (1) recurrent abdominal pain is not usually a reason for seeking medical attention among people with low socioeconomic status. (2) patients with a lower socioeconomic status who are referred to the Gastroenterology unit, who are rarely assisted due to monetary limitations, and (3) people belonging to the middle class who used to pay for private medical care attention, have turned to public health care centers due to the fact that economic conditions have become impaired in Argentina during the last years.

The factors associated with *H. pylori* positivity were the number of siblings, presence of cats and birds in the house, and antecedents of gastritis among family members (Table 2). After binary logistic regression analysis, only the number of siblings and contact with pet cats remained factors for increasing risk of *H. pylori* infection.

The observation that the presence of cats in the house may increase the probability of being positive for *H. pylori* infection in children with gastrointestinal symptoms require further investigation, especially because controversial results have been reported for the role of cats and other domestic pets in association with *H. pylori*^[6,15-18]. Cats are commonly infected with gastric *Helicobacter*-like organisms (GHLOs) as “*H. heilmannii*”, that might be transmitted to humans^[19-21]. Therefore, a positive Urea Breath Test result in a patient could represent a gastric presence of urease positive *Helicobacter* species other than *H. pylori*. However, as prevalence of “*H. heilmannii*”-like organisms in humans is relatively low, this topic requires further clarification.

Low socioeconomic status and poor sanitary standards were described as risk factors for the acquisition and transmission of *H. pylori*^[5-7]. Given that most of the studied population had good socioeconomic conditions, we could not demonstrate an association between *H. pylori* status, socioeconomic conditions and sanitary standards. It is important to point out that the correlation coefficients depends strongly on sample sizes and balance between them^[22]. Another factor associated with *H. pylori* infection in the studied population was the history of gastritis among family members. In contrast, other authors have not found a correlation between family history of gastric disease and *H. pylori* infection in the children^[6,17]. Major limitations in the association of clinical history with *H. pylori* status are, first, the definition used to establish gastric diseases and second, the technique employed to diagnose *H. pylori* infection.

In conclusion, we found that prevalence of *H. pylori* infection in children with upper gastrointestinal symptoms referred to a Gastroenterology service in the Province of Buenos Aires, Argentina was 40%, similar to the prevalence reported in developed countries. In addition, number of siblings in the household and presence of pet cats are predicting variables for *H. pylori* colonization. Our study

provides important information regarding the prevalence of *H. pylori* infection in symptomatic children in Buenos Aires, Argentina, and factors associated with increasing risk for *H. pylori* positivity in a developing country.

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Treatment of gastric precancerous lesions with Weiansan

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CONCLUSION: WAS improves clinical symptoms by suppressing GA, IM and dysplasia and eliminating *H pylori*.

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Key words: Weiansan; Gastric precancerous lesions; Clinical observation; *H pylori* elimination

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Abstract

AIM: To observe the curative effect of Weiansan (WAS) on gastric precancerous lesions (GPL) and *H pylori* elimination.

METHODS: Seventy-six patients with GPL were randomly divided into two groups: WAS group ($n = 42$) and Weifuchun (WFC) group ($n = 34$). The patients in the WAS group were administered 5 g WAS 3 times a day, and the patients in the WFC group took WFC (4 tablets) 3 times a day. To monitor inflammation of gastric mucosa, degree of glandular atrophy (GA), intestinal metaplasia (IM) and dysplasia, and *H pylori* infection, all patients underwent gastroscopy and biopsy with pathological examination before and after treatment. Fifty male Sprague-Dawley (SD) rats were used in animal experiments. Of these, 10 served as the control group ($n = 10$), 40 were given ranitidine combined with *N*-methyl-*N*¹-nitro-*N*-nitrosoguanidine (MNNG) for 12 wk and divided into 4 groups randomly: model group ($n = 10$), high-dose WAS group ($n = 10$), low-dose WAS group ($n = 10$) and WFC group ($n = 10$). Twelve weeks later, all rats were killed and a 2 cm × 1 cm tissue was taken from the lesser curvature of the gastric antrum. *H pylori* infection was determined by the fast urease method.

RESULTS: The curative effect in WAS groups was similar to that in WFC groups. There was no statistical difference in degree of GA, IM and dysplasia between WAS and WFC groups. The rate of *H pylori* infection in the model group (positive/negative: 9/1) was significantly higher than that in the control group (positive/negative: 1/9) ($P < 0.01$). *H pylori* elimination in the high-dose WAS group (positive/negative: 4/6) and low-dose WAS group (positive/negative: 6/4) was similar to that in the WFC group (positive/negative: 4/6) ($P > 0.05$).

INTRODUCTION

Gastric precancerous lesion (GPL) is one of the diseases of the digestive system, easily occurring in gastric mucosa with changes such as gastric epithelial dysplasia (GED) and intestinal metaplasia (IM) resulting in chronic atrophic gastritis (CAG)^[1]. Traditional Chinese medicine (TCM) can prevent and cure GPL, and has become one of the hotspots in research^[2]. In order to find its accurate and effective therapy, GPL was treated with Weiansan (WAS) and its effect on eliminating *H pylori* was evaluated.

MATERIALS AND METHODS

Patients

Seventy-eight patients were diagnosed as insufficiency of the spleen- and stomach-qi or stagnation of qi and stasis of blood. CAG was confirmed by gastroscopy. The patients were randomly divided into two groups: WAS group ($n = 42$) and Weifuchun (WFC) group ($n = 34$).

The WAS group included 32 males and 10 females aged 24-69 years with a mean age of 40 ± 2.1 years. Of the 42 patients in this group, 20 had mild CAG and 22 had moderate to severe CAG.

The WFC group included 24 males and 10 females aged 25-68 years with a mean age of 41 ± 2.3 years. Of the 34 patients in this group, 14 had mild CAG and 20 had moderate to severe CAG. There was no significant difference between the two groups.

Experimental animals

Fifty 15-d old male Sprague-Dawley rats weighing

Table 1 Changes of GA in WAS and WFC groups after treatment

Group	Cases (n)	Atrophy improvement			No change	Severe
		0	+	++<-		
WAS	42	2	22	6	10	2
WFC	34	2	16	8	8	0

0: Have changes but not achieve a state (less than a +); +: Effective; ++<-: Obviously effective.

100-150 g were bought from Experimental Animal Centre, Chinese Academy of Medical Sciences. The feed was purchased from Keao Xieli Feed Limited Company.

The evaluation standard of clinical curative effects was drawn up according to the 2nd version of "Clinical Research Guiding Principle of New Medicine of TCM" and other corresponding reports^[3].

Disappearance of clinical symptoms and active inflammation, improvement of chronic inflammation, reduction of GA, IM and gastric epithelial dysplasia (GED) were judged as curative. Disappearance of main clinical symptoms, improvement of acute and chronic inflammation, two-degree reduction of GA, IM and GED were judged as obviously effective. Improvement of clinical symptoms, one-degree reduction of GA, IM and GED were judged as effective. No improvement of clinical symptoms and pathological change was judged as ineffective.

Clinical research method

To observe the mucous membrane inflammation, degree of GA, IM and GED, all the patients underwent gastroscopy and pathological examination before and after 24 wk of treatment. The rate of *H pylori* infection was also determined. The patients in WAS group received 5 g WAS 3 times a day, while the patients in WFC group were given 4 tablets of WFC 3 times a day for 12 wk as a course of treatment. The two groups received two courses of the treatment.

Experimental method

One week after increasing compatibility, the rats drank MNNG (50 µg/L) and were fed standard powder feed containing 0.03% ranitidine for 12 wk as previously described^[4]. Ten rats in the control group were fed normally. After administration of MNNG for 12 wk, the other 40 rats were divided into 4 groups, 10 rats in each group. The rats in the model group were given physiological saline *via* gavages (1 mL/100 g) once a day; the rats in the high-dose WAS group were physiological saline *via* gavages (5 g*20/65 g body weight); the rats in the low-dose WAS group and WFC group were fed by gavages individually (1 mL/100 g) once a day. After administration of WAS and WFC for 12 wk, all the rats in the 5 groups were killed and their stomachs removed. The gastric cavity was cut open along the greater curvature, cleaned and fixed in buffered formalin solution. A 2 cm × 1 cm tissue was removed from the lesser curvature of the gastric antrum. *H pylori* situation was determined by the

Table 2 Changes of GED and IM in WAS and WFC groups after treatment

Group	Cases (n)	GED and IM improvement			No change	Severe
		0	+	++<-		
WAS	20	2	8	2	4	2
WFC	16	0	4	6	4	2

0: Have changes but not achieve a state (less than a +); +: Effective; ++<-: Obviously effective.

fast urease method. The *H pylori*-eliminating effect in the 5 groups was compared.

Statistical analysis

The pairs-*t* test was applied to the data before and after the treatment, the ratio was examined with chi-square test, and the rank material was examined with Ridit. All analyses were performed with SPSS 10.0. *P* < 0.05 was considered statistically significant.

RESULTS

Effect of WAS and WFC on glandular atrophy

The degree of GA, IM and GED was classified into mild, moderate and severe grades according to gastroscopy findings. The patients received gastroscopy and pathological examination respectively before and after 24 wk of treatment. Improvement of clinical symptoms was analyzed. According to the evaluation standard, clinical curative effects were judged as curative, obviously effective, effective and ineffective, respectively.

The results suggested that the above-mentioned indexes had no significant difference between the WAS and WFC groups before and after treatment (*P* > 0.05). After treatment, such indexes were improved both in the WAS group and in the WFC group (*P* < 0.05), suggesting that both WAS and WFC could improve GA, IM and dysplasia. The total effective rate for alleviating the GA degree was 71.43% in the WAS group and 76.47% in the WFC group, respectively (total effective rate for alleviating the GA degree = the alleviated GA degree/total cases × 100%).

The total effective rate for alleviating the GED and IM degree was 60.00% in the WAS group and 62.50% in the WFC group, respectively (total effective rate of alleviating the GED and IM degree = the alleviated GED and IM degree/total cases × 100%). The histological changes in GED and IM were not obvious in both groups after treatment. It might be due to a too short period of treatment, concentration of medicine, and combined herbal medicine treatment.

Elimination of *H pylori* in patients after treatment with WAS and WFC

Before treatment, *H pylori*-infected patients were examined in both groups. After treatment, the eradication rate of *H pylori* in the WAS group was higher than that in the WFC group (*P* < 0.05), indicating that the *H pylori*-eliminating effect in WAS group was better than that in the WFC group (Table 1, Table 2, Table 3, Table 4).

Table 3 Curative effect in WAS and WFC groups

Group	Cases (n)	cure	Obviously effective	Effective	Ineffective	Effective rate (%)
WAS	42	4	20	12	6	85.71
WFC	34	4	12	12	6	82.35

Table 4 *H. pylori* elimination in WAS and WFC groups

Group	Cases (n)	Positive	Negative	Negative rate (%)
WAS	42	6	36	85.74 ^a
WFC	34	13	21	61.71

^a*P* < 0.05 vs WFC group.

H. pylori situation in animal experiment and eliminating effects of WAS and WFC

Compared with the control group (positive/negative: 1/9), the *H. pylori* situation in the model group (positive/negative: 9/1) was noticeably better (*P* < 0.01). The *H. pylori*-eliminating effect in the high-dose WAS group (positive/negative: 4/6) and low-dose WAS group (positive/negative: 6/4) was similar to that in the WFC group (positive/negative: 4/6) (*P* > 0.05). The eradication rate of *H. pylori* in the three groups was obviously higher than that in the model group (*P* < 0.05, Tables 5 and 6).

DISCUSSION

GPL belongs to the “stomach distension” and “epigastralgia” category in traditional Chinese medicine. It results from spleen and stomach injury, blood stasis due to impeding circulation of qi and blood caused by improper diet, the 6 abnormal climatic factors and the 7 abnormal emotions. The keys are spleen deficiency, qi stagnation and blood stasis. The main local symptoms are distension and fullness of the stomach, belches and poor appetite, constipation or diarrhoea. The general symptoms are lassitude and weakness, dizziness and emaciation, sallow complexion, pale and dark tongue. The basic factor and pathogenesis are deficiency in origin and excess in superficiality, and complicated syndromes of deficiency and excess. Therefore it should be treated both causally and symptomatically. The corresponding principle of WAS is to strengthen the spleen, replenish and regulate qi, invigorate blood. Our clinical study demonstrated that the total effective rate for GPL was about 85.70%, indicating that WAS plays a certain role in blocking GPL. WAS is composed of astragalus root, pilose asiabell root, bighead atractylodes rhizome, cinnamon bark, red sage root, scorpion, rhizome corydalis, fructus anrantii, processed grain and wheat, *etc.*

In the decoction, astragalus root, pilose asiabell root, bighead atractylodes rhizome, and cinnamon bark increase the gastric mucosa barrier, enhance the immunity, strengthen the phagocytic function of the reticuloendothelial system, elevate the lymphocyte transformation rate and promote anti-body formation.

Table 5 *H. pylori* situation in GPL of model and control groups after 12 wk of treatment

Group	Cases (n)	Positive	Negative	Infective rate (%)
Model group	10	9	1	90 ^a
Control group	5	0	5	0

^a*P* < 0.01 vs control group.Table 6 *H. pylori* eliminating situation after 12 wk of treatment

Group	Cases (n)	Positive	Negative	Eliminating rate (%)
High-dose WAS group	10	4	6	60
Low-dose WAS group	10	6	4	40
WFC group	10	4	6	60
Model group	10	9	1	10

Red sage root, scorpion, and rhizome corydalis containing a broad spectrum of anti-neoplastic components, may invigorate the blood to remove blood stasis, relieve spasm and pain, inhibit tumour occurrence, improve the gastric mucosa blood circulation, accelerate topical inflammation absorption, help atrophic glands to restore and remove the deleterious substance in epithelial cells. Fructus anrantii, processed grain and wheat can relieve depression of liver-qi, activate qi and promote digestion^[5]. These herbal medicines cooperate in harmony and exert a major effect on GPL.

A total of 7496 cases related to CAG have been reported, and the insufficiency type of the spleen and stomach ranks first^[6], indicating that insufficiency of the spleen correlates positively with the severity of gastric diseases. The *H. pylori* infection rate and the degree of infection in patients with qi-stagnation and middle-warmer deficiency are remarkably different from those in patients with other types of CAG, suggesting that the *H. pylori* infection rate is closely related to insufficiency of the spleen which has become the most important factor for stomach cancer development. Therefore insufficiency of the spleen and qi stagnation should be closely monitored because of their high carcinogenic tendency. Our study has confirmed that strengthening the spleen can improve GPL, reduce gastric mucosa inflammation, help atrophic glands to regenerate and restore, reverse and restore gastric mucosa dysplasia and IM, promote differentiation and maturation of atypical cells to normal cells. Drugs strengthening the spleen increase the gastric mucosa barrier effect and protect carcinogens from attacking by enhancing immunity, strengthening the phagocytic function of the reticuloendothelial system and promoting the lymphocyte transformation rate. All these indicate that the method of strengthening the spleen can prevent GPL, and this method has become one of the therapies for preventing and treating gastric mucosa precancerous lesions^[7,8].

In addition, studies also indicate^[9-11] that microcirculation dysfunction is generally followed by CAG. The

common clinical symptoms include fixed stomach pain and purple dark or dark red or pale dark or ecchymosed tongue. In our study, gastroscopy demonstrated that the degree of GA, IM and GED in blood stasis type of GPL was more severe than that in other types, suggesting that the mechanism underlying blood stasis exists in GPL. Gastric mucosa dysplasia and IM highly correlate with microcirculation. Blood-invigorating herbal medicines can remove blood stasis and protect gastric mucosa by improving GPL, gastric mucosa microcirculation, establishing collateral circulation, improving local ischemia and hypoxia, increasing gastric mucosa blood flow, reducing inflammation and helping atrophic glands to regenerate. Therefore invigorating the blood to remove blood stasis has also become the key to preventing and treating gastric mucosa precancerous lesions.

In conclusion, according to the pathogenesis of insufficiency of the spleen and blood stasis, the therapy for GPL can be established. Treatment of PGL with WAS is to strengthen the spleen, replenish and invigorate qi and blood. Furthermore, WAS can improve the clinical symptoms of GPL by suppressing GA, IM and dysplasia and eliminating *H pylori*.

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Colonic metastasis of Klatskin tumor: Case report and discussion of the current literature

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Abstract

We report the case of a 65-year old male patient who initially presented with recurrent episodes of upper abdominal pain, lack of appetite and weight loss. Abdominal ultrasound indicated enlarged intrahepatic bile ducts, abdominal CT scan and ERC were performed and bile duct carcinoma (Klatskin Type III b) was diagnosed. The tumor was located in the segments 2,3,4 and 1 with possible invasion of the left intrahepatic portal vein. Both the segments 2 and 3 of the liver were atrophic and displayed a cholestatic bile duct system. Preoperatively an intraductal stent was placed in the left bile duct using ERC to drain the left hepatic lobe. A specimen of the ascites present preoperatively displayed no malignant cells. After evaluation of the preoperatively obtained data left hepatic resection was planned. Following laparotomy we found local peritoneal carcinosis in the ligamentum hepatoduodenale with lymphatic nodules that tested positive for cholangiocellular carcinoma in online pathological examination. In the course of further exploration of the abdomen a solid tumor was detected in the sigmoid colon. Regarding the advanced stage of the neoplasm it was decided to cancel hemihepatectomy and perform sigmoid resection only in order to guarantee uncomplicated intestinal passage. The sigmoid colon was removed by a typical resection technique with end-to-end anastomosis. Histological examination of the resected sigmoid revealed transmural manifestation of a malignant neoplastic process with both a tubular and a solid growth pattern in conformity with metastasis of a Klatskin tumor. The mucosal layer showed no neoplastic alteration. Peritoneal carcinosis is a common phenomenon in the dissemination pattern of advanced-stage Klatskin tumors, yet to our knowledge this is the first case of intramural colonic growth following peritoneal metastasis.

INTRODUCTION

Bile duct tumors are rare neoplasms with an incidence of 0.5-1/100 000^[1]. Predisposing factors include mainly PSC with a thirty-fold increased risk as well as choledochal cysts and parasitic infections (*clonorchis sinensis*, *opisthorchis viverrini* et *felinus*)^[1,2]. These tumors usually present with clinical symptoms such as upper right abdominal pain, weight loss or jaundice at a rather advanced stage when the process has already grown to a rather significant extent. The tumor type and localisation are categorized according to the Bismuth criteria^[3]. After quite a sceptical approach towards surgical therapy for Klatskin tumors until the early 1990s, during the last years surgical results have demonstrated that even patients with a locally advanced stage of proximal bile duct carcinoma without distant metastasis profit from surgical resection^[3,4,5]. Today 5-year survival rates from 10% to 40% are reported with even higher survival rates in specialized centers^[6,7,8].

CASE REPORT

We report the case of a 65-year old male patient who had experienced episodes of upper abdominal pain for several months. When presentation at our institution he did not develop jaundice, bilirubin was 0.6 mg/dL after stent-placement in the left hepatic duct prior to admittance to our institution.

Due to lack of appetite he had lost five kilograms over the last four months. Laboratory values at admission time were as follows: total bilirubin 0.6 mg/dL, conj. 0.4 mg/dL, AST 75 U/L, ALT 39 U/L, gGT 63 U/L, AP 82 U/L, lipase 210 U/L, amylase 278 U/L, leukocytes 9.29/nL, thrombocytes 206/nL, Hb 11.5 g/dL, TPZ 83%, INR 1.15 CEA 2.6 µg/L, AFP 29 µL/L, CA 19-9 803 U/mL.

Clinical examination revealed a man of reduced physical fitness considering his age and upper right abdominal pain at palpation. After abdominal ultrasound indicating enlarged intrahepatic bile ducts a CT scan was

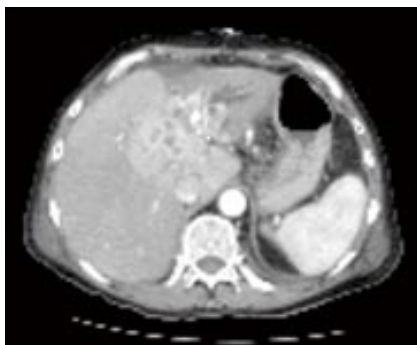


Figure 1 CT scan shows Klatskin tumor type III b with intrahepatic bile duct enlargement, cholestasis and atrophy of the left hepatic lobe.

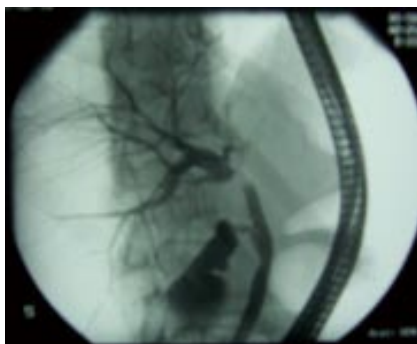


Figure 2 ERC displays good filling of the right intrahepatic bile duct system whereas the left system could not be displayed due to tumorous stenosis in the distal part of the ductus hepaticus sinister reaching into the ductus hepaticus communis.

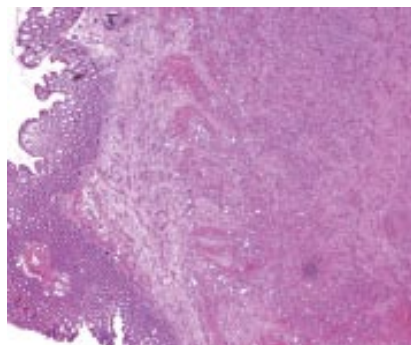


Figure 3 Histological examination of the resected colonic neoplasm reveal intramural metastasis of adenocarcinoma infiltrating the muscular and submucosal layers.

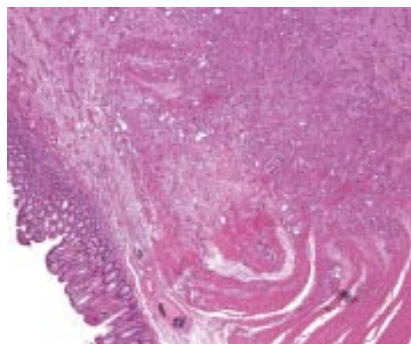


Figure 4 Histological examination of the resected colonic neoplasm reveals intramural metastasis of adenocarcinoma infiltrating the muscular and submucosal layers.

performed demonstrating a mass of hypervascularized tissue in the central liver hilum within the segments 2, 3, 4 and 1. Enlarged lymphatic nodes in the hepato-duodenal ligament could also be detected. The left intrahepatic bile duct system proximal of the tumor was markedly enlarged with signs of massive cholestasis.

CT scan showed Klatskin tumor Typ III b growth with intrahepatic bile duct enlargement, cholestasis and atrophy of the left hepatic lobe (Figure 1).

An endoscopic retrograde cholangiography (ERC) was then conducted to examine the detailed bile duct anatomy. This displayed good filling of the right intrahepatic bile duct system whereas the left system could not be displayed due to tumorous stenosis in the distal part of the ductus hepaticus sinister reaching into the ductus hepaticus communis (Figure 2). After careful preoperative evaluation the patient was subjected to surgical resection. At the start of the surgical procedure multiple positive lymphatic nodules were detected in the hepatoduodenal ligament. While further exploring the peritoneal cavity for potential additional manifestations of the tumor a solid mass could be found in the sigmoid colon. As this was a preoperatively unknown process which appeared highly suspicious to be of neoplastic origin, it was decided to resect this segment of the colon. Sigmoid resection was performed using the standard no-touch technique. The resected sigmoid colon was then subjected to histological examination: Histomorphology and immune-histochemistry revealed an intramural colonic metastasis of a badly differentiated adenocarcinoma of the bile duct displaying solid as well as tubular segments of growth with a large number of mitotic cells. No neoplastic alteration of the colon mucosa could be detected.

A separately growing sigmoid neoplasm of colonic

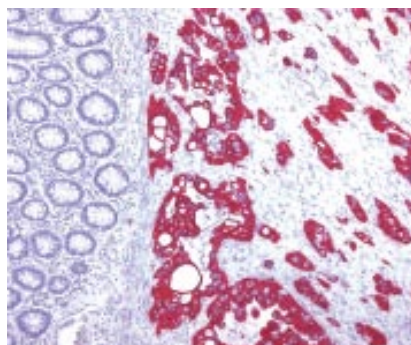


Figure 5 Immunohistological staining shows adenocarcinoma of the non-intestinal klatskinoid type.

origin could be ruled out (Figures 3, 4). Histological examination of the resected colonic neoplasm revealed intramural metastasis of adenocarcinoma infiltrating the muscular and submucosal layers (Figure 5). Immunohistological staining showed adenocarcinoma of the non-intestinal klatskinoid type.

DISCUSSION

As far as our clinical experience and the extensive literature research are concerned, this is the first case ever reported of colonic metastasis of Klatskin type bile duct carcinoma.

Lymphatic metastasis of Klatskin tumors into lymph nodes along the hepatoduodenal ligament is a common and well-known phenomenon as is intrahepatic dissemination to areas distant from the original tumor location^[1,3,6]. In more advanced stages of the disease we have also found peritoneal carcinosis and lung metastasis. Intestinal dissemination of a Klatskin tumor has not been reported up to date.

As careful pathological inspection-revealed colonic

metastasis is not merely due to peritoneal carcinosis spreading to the serosa of the sigmoid colon but originates within the intestinal wall.

In the advanced stage of bile duct carcinoma, peritoneal carcinosis leads to spreading of neoplastic cells within the abdominal cavity and consecutive tumor growth in multiple and various locations. Furthermore dissemination of cancer cells during surgery for bile duct carcinoma can lead to tumor cell spreading followed by metastasis in surgical scars^[9,10]. Whereas these phenomena lead to malignant cell growth originating from the serosal layer where metastatic cells are initially implanted, our case describes the continuing growth of disseminated bile duct carcinoma cells into and within the intestinal wall of the sigmoid colon.

While this case remains unique up to date, it may suggest to include colonoscopy in the preoperative staging procedures in selected cases.

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CASE REPORT

Multiplex neuritis in a patient with autoimmune hepatitis: A case report

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Abstract

A 37-year old woman presented with a 9-year history of hepatitis of unknown origin and aminotransferases within a 3-fold upper limit of normal. Autoimmune hepatitis (AIH) was diagnosed on the basis of elevated aminotransferases, soluble liver antigen/liver pancreas (SLA/LP) autoantibodies and characteristic histology. Immunosuppressive therapy led to rapid normalization of aminotransferases. Two years later, the patient developed left sided hemisensory deficits under maintenance therapy of prednisolone and azathioprine (AZT). Later she developed right foot drop and paraesthesia in the ulnar innervation territory on both sides. Magnetic resonance imaging (MRI) and cerebral panangiography suggested cerebral vasculitis. Neurological investigation and electromyography disclosed multiplex neuritis (MN) probably due to vasculitis. Consistent with this diagnosis, autoantibodies to extractable nuclear antigens were detectable in serum. Immunosuppression was changed to oral 150 mg cyclophosphamide (CPM) per day. Prednisolone was increased to 40 mg/d and then gradually tapered to 5 mg. Oral CPM was administered up to a total dose of 40 g and then substituted by 6 times of an interval infusion therapy of CPM (600 mg/m²). Almost complete motoric remission was achieved after 3 mo of CPM. Sensibility remained reduced in the right peroneal innervation territory. Follow-up of cranial MRI provided stable findings without any new or progressive lesions. This is the first report of multiplex neuritis in a patient with autoimmune hepatitis.

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Key words: Multifocal peripheral neuropathy; Immu-

INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic autoimmune disease of the liver of unexplained aetiology associated with autoantibodies^[1]. Overlapping syndromes with other autoimmune liver diseases, such as primary biliary cirrhosis or primary sclerosing cholangitis are increasingly reported. AIH can also be associated with extra-hepatic autoimmune diseases such as Graves' disease, Hashimoto thyroiditis, mixed connective tissue disease^[2], membranous glomerulonephritis^[3], Sjogren's syndrome^[4] and more often with systemic lupus erythematosus (SLE)^[5]. While SLE is often associated with neuropathic symptoms, only four cases of neuropathy in AIH are published to date.

Multiplex neuritis (MN) results from multifocal injury of the peripheral nerves. Causative processes include vasculitis (frequently related to diabetes or polyarteritis nodosa), sarcoidosis, lymphoma and peripheral nerve tumours^[6].

We observed a patient with AIH who developed MN due to vasculitis during combined immunosuppressive treatment with corticosteroids and azathioprine two years after AIH diagnosis. Treatment with cyclophosphamide led to remission of both conditions.

CASE REPORT

A 37-year old woman presented in June 2000 with a 9-year history of hepatitis of unknown origin and aminotransferases ranging from 70 U/L to 100 U/L (2-3 × ULN). Two years before presentation, antibodies to TPO were found, but the patient since then was still euthyretic. Except for that, she had no previous medical history and was asymptomatic. Physical examination showed no pathological findings. She had no history of alcohol abuse and did not commonly use any medications. She had no neurological symptoms. At presentation, aspartate aminotransferase (AST; 37 U/L, normal value < 32 U/L) and alanine aminotransferase (ALT; 54 U/L,

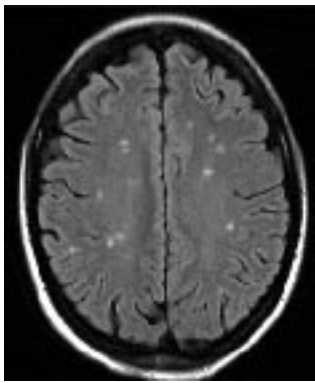


Figure 1 MRI (T2w/FLAIR) of the centrum semiovale showing multiple foci of subcortical signal enhancements which are characteristic but not specific for vasculitis.

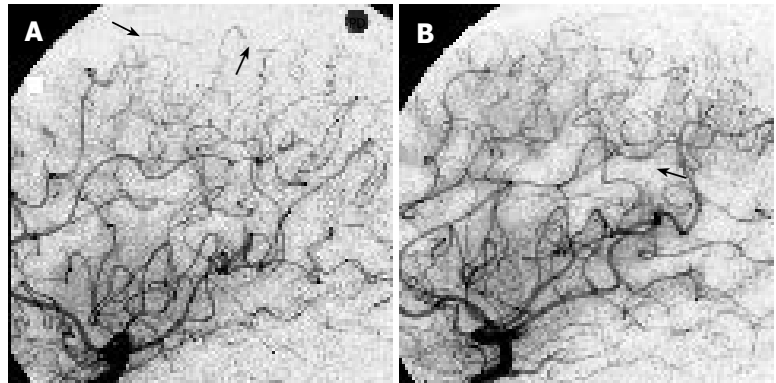


Figure 2 Left internal carotid artery (ACI) demonstrating irregularities of the distal parts of branches of the callosomarginal artery (arrows) (A) and right ACI demonstrating the break of a central branch of the middle cerebral artery (arrow) (B).

normal value < 31 U/L) were slightly elevated. In contrast, gamma-glutamyltransferase (gamma-GT; 19 U/L, normal value ranging 5-36 U/L), alkaline phosphatase (AP; 123 U/L, normal value ranging 70-180 U/L), total bilirubin (0.24 mg/dL, normal value < 1.2 mg/dL), total serum protein (79 g/L, normal value ranging 60-80 g/L), albumin (65.5%), gamma-globulin (16.4%) and serum IgG (13.4 g/L) were normal. Complete peripheral blood count, blood urea nitrogen and creatinine levels were normal. Testing for hepatitis B surface antigen (HBsAg) and antibodies to hepatitis B core antigen (HBcAb) was negative, testing for antibody to hepatitis C virus (HCV) by ELISA and HCV-RNA by polymerase chain reaction was also negative. Serum levels of copper and ceruloplasmin were normal. Autoantibodies to soluble liver antigen/liver pancreas (SLA/LP) were found at a titre of > 200 U/mL by ELISA. The presence of SLA/LP autoantibodies was confirmed by immunoblot. At that time no other autoantibody was found. Liver biopsy showed mild periportal hepatitis, some areas showed dissection of the liver parenchyma by inflammatory cells with hepatocyte ballooning and rosetting.

Based on these findings, the diagnosis of SLA/LP positive AIH was made. Treatment with prednisolone at a daily dose of 20 mg was initiated and due to a quick and complete normalization of aminotransferases, prednisolone could be gradually tapered to a maintenance dose of 5 mg per day. Six months later, treatment was tapered out, but the disease slowly relapsed with a mild elevation of liver enzymes and progressive fatigue. Therefore, immunosuppressive treatment was readjusted to a daily dose of 40 mg prednisolone, which was then reduced weekly down to a maintenance dose of 10 mg per day. Serum aminotransferase levels rapidly normalized and clinical symptoms disappeared. Azathioprine was added at a dose of 75 mg per day and increased to 100 mg per day after two weeks.

In the following months, complete remission of AIH was maintained by medication with 7.5 mg/d prednisolone and 100 mg/d azathioprine. At this stage, the patient developed acute neurological disturbances, initially with a left sided hemisensory deficit. A few weeks

later, the patient developed paraesthesia in both ulnar innervation territories and subsequently a right sided peroneal paresis and mild weakness of hip flexion. She never had any lumbar pain. An infection with *Borrelia burgdorferi* and *Treponema pallidum* was ruled out by the absence of antibodies. MRI of the brain showed multiple paraventricular lesions, which were assumed to be of vasculitic character (Figure 1). Angiography of the brain showed irregularities of intracerebral arteries (Figure 2) consistent with vasculitis. Cerebrospinal fluid examination and electrophysiological examination (evoked potentials) were normal. Electromyography showed acute denervation in the anterior tibialis muscle and to a lesser degree in the medial proportion of the thigh muscle. To exclude radicular lesions, MRI of the spinal cord was performed, which showed distant degenerative changes, but no pathology on the right-sided L4 and L5 nerve roots. Accordingly, neurological examination revealed no loss of deep tendon reflexes or radicular pain. History, clinical and neurophysiological examination, cerebral angiography and MRI together with the emergence of autoantibodies to Ro/SSA supported the clinical diagnosis of MN, due to vasculitis of the nervous system.

For the treatment of vasculitis, azathioprine was replaced by cyclophosphamide (CPM) at an initial dose of 150 mg/d given orally. CPM is also effective in the treatment of AIH^[7]. Prednisolone was increased to 40 mg/d and then gradually tapered to 5 mg/d. According to the guidelines for the treatment of isolated vasculitis of the nervous system, oral CPM was administered at a total dose of 40 g. Thereafter, CPM was given 6 times by infusion therapy (600 mg/m²) at an 8-wk interval. Total recovery of the motoric deficits and almost complete recovery of the sensory deficits were achieved after 3 mo. Sensibility was only diminished in the peroneal nerve segment of the right leg. Cranial follow-up MRI, performed 6 mo after the initiation of CPM provided stable findings. The patient was in the fifth year of follow-up after AIH diagnosis and was receiving maintenance therapy consisting of 5 mg prednisolone and 100 mg azathioprine, AIH was still in remission and the patient remained free of further neurologic symptoms at the time she reported.

DISCUSSION

Multiplex neuritis (MN) occurs as a complication of vasculitis but can also develop in toxic or metabolic processes. Small vessel vasculitis typically affects the 50-400 micron vessels of the vasa nervorum, leading to randomly distributed ischemia along the nerve^[8]. This, in turn, may lead to a distinctive clinical picture of scattered neurological symptoms, which can be attributed to multiple complete or incomplete peripheral nerve or nerve root lesions. It is reported that more than half of vasculitis patients present with MN^[9]. The most frequent single nerve lesion is the peroneal nerve paresis^[10].

To date multiplex neuritis in hepatic diseases has been reported as a rare complication in patients with chronic hepatitis B^[11-13], in one patient with acute hepatitis A infection^[14] and in patients with hepatitis C and associated cryoglobulins^[15]. This latter manifestation is likely due to the cryoglobulinemic vasculitis seen in hepatitis C. The pathogenesis of this neuropathy in viral hepatitis without cryoglobulins has not yet been fully elucidated. It has been suggested that nerve lesions result from viral factors directly or from deposits of immune complexes in the vasa nervorum, which then may cause vasculitis and ischemia of the nervous fibers, as it has been shown for cryoglobulinemic neuropathies^[11,12,16].

Neuropathies in AIH patients are an extremely uncommon finding. Four patients have been described in the literature suffering from AIH and peripheral neuropathy^[17,18] and one patient suffering from AIH and cranial neuropathy, but this patient also had positive HCV serology and polymyositis^[19]. The patient described here suffered from clearly defined SLA/LP-positive AIH without any evidence for viral infection. Her asymmetric sensory and motor symptoms of both central and peripheral nervous system origin, with a clear stepwise onset developing over months, suggested vasculitis of the nervous system, which was compatible with the multifocal peripheral nerve involvement and ischemic brain lesions on MRI. Cerebral angiography provided objective evidence for nervous system vasculitis, and neurophysiological studies confirmed multiple peripheral nerve involvement.

To our knowledge, this is the first reported patient with AIH associated with vasculitic MN. Our report indicates that MN can develop in non-viral hepatitis. Both AIH and vasculitic MN are autoimmune systemic disorders of unknown origin, which occurred sequentially in our patient, suggesting that MN seems to develop independently of AIH. In our patient, the disease activity of AIH was not associated with that of MN, and MN developed despite immunosuppressive therapy with 7.5 mg prednisolone and 100 mg azathioprine per day, suggesting that MN may need higher levels of immunosuppression for remission than AIH.

In conclusion, medication with cyclophosphamide in combination with intensified prednisolone therapy can rapidly improve neurological symptoms and offer an

option for the prevention of further nerve injury.

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A left-sided periappendiceal abscess in an adult with intestinal malrotation

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Abstract

Left-sided periappendiceal abscesses occur in association with two types of congenital anomaly: intestinal malrotation and situs inversus. It is difficult to obtain an accurate preoperative diagnosis of these abscesses due to the abnormal position of the appendix. We present an unusual case of a left-sided periappendiceal abscess in an adult with intestinal malrotation, the diagnosis of which was a challenge.

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Key words: Left-sided periappendiceal abscess; Intestinal malrotation; Diagnosis

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INTRODUCTION

Intestinal malrotation is a congenital anomaly referring to either nonrotation or incomplete rotation of the primitive intestinal loop around the axis of the superior mesenteric artery during fetal development. While most cases of intestinal malrotation present with bilious vomiting in the first month of life^[1], rare cases present in adulthood^[2]. It is important that physicians be aware of the possibility of

this disease when treating adult patients with abdominal pain because diagnosis of intestinal malrotation can be difficult. The present report details an unusual case of a left-sided periappendiceal abscess with intestinal malrotation in an adult who presented with a painful mass in the left lower quadrant.

CASE REPORT

A 43-year-old man with no previous abdominal complaints presented to the emergency room of our hospital with a 2-wk history of a painful mass in the left lower quadrant. Previously, his physician had administered intravenous antibiotics for suspected diverticulitis, but increasing abdominal pain led to his hospital presentation. Physical examination revealed a body temperature of 37.4°C and an 8 cm-sized painful mass in the left lower quadrant. Laboratory tests showed a normal white cell count ($4.6 \times 10^9/L$) and an elevated C-reactive protein concentration (104 mg/L). Computed tomography (CT) abdominal scanning revealed a solid fluid-containing tumor in the left lower quadrant (Figure 1), suggesting an inflammatory mass with abscess formation. We drained the abscess using percutaneous drainage (PCD) and administered intravenous antibiotics while trying to determine the exact cause of the abscess. One week later, the patient had a soft abdomen with no specific complaint.

To identify the cause of the abscess, we performed a contrast enema with gastrograffin. This procedure revealed that a fistulous tract of the colon did not exist, and the entire colon was seen in the left half of the abdomen with the cecum in the left lower quadrant (Figure 2). Contrast filling of the terminal ileum was apparent. An irregular contour of the cecum was also observed, with no contrast filling of the appendix. Figure 2 also shows a pig-tail catheter located in the area corresponding to an abscess pocket. With this information, the abdominal CT was again reviewed. Further review indicated a periappendiceal abscess with intestinal malrotation due to the anatomic location of the right-sided small bowel, left-sided large bowel, the abnormal position of the superior mesenteric vessels, and an inflammatory mass in the ileocecal area.

Surgery was performed through a lower midline incision after obtaining informed consent. Surgical findings confirmed the severe inflammatory changes of the appendix and ileocecal region located in the left lower quadrant (Figure 3). An ileocecectomy was performed. Pathology testing indicated gangrenous appendicitis with severe peri-

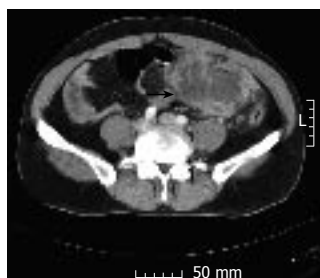


Figure 1 CT abdominal scan showing the presence of a solid fluid-containing tumor in the left lower quadrant (arrow).



Figure 2 Radiograph using water-soluble contrast media showing the entire colon present in the left half of the abdomen, with the cecum in the left lower quadrant. Contrast filling of the terminal ileum is shown (arrow). Irregular contour of the cecum (arrowhead) is shown with no contrast filling of the appendix. A pig-tail catheter is located at the area corresponding to the abscess pocket.



Figure 3 Intraoperative photography showing severe appendix inflammation (arrow) and the ileocecal region located in the left lower quadrant.

cecal inflammation. The patient recovered uneventfully after surgery.

DISCUSSION

Intestinal malrotation is a congenital anomaly referring to either non-rotation or incomplete rotation of the primitive intestinal loop around the axis of the superior mesenteric artery during fetal development. As most complications associated with intestinal malrotation occur in the first month of life^[1], this disease may not be foremost in the mind of physicians with exclusively adult patients. However, all such physicians should be familiar with intestinal malrotation owing to its associated diagnostic difficulty and the devastating consequences of failure of recognition^[2]. In general, adults with intestinal malrotation present

in one of three ways^[3]. Some patients present with acute obstructive symptoms and signs of impending abdominal catastrophe. Others present with chronic abdominal complaints that include both pain and intermittent obstruction. Lastly, some present with atypical symptoms common to abdominal diseases unrelated to intestinal malrotation, such as in the present report.

The increasing use of abdominal CT as the first imaging modality in patients with various abdominal complaints means that the importance of identifying malrotation by CT cannot be overemphasized. Malrotation can be diagnosed on CT by the anatomic location of a right-sided small bowel, a left-sided colon, an abnormal relationship of the superior mesenteric vessels, and aplasia of the uncinate process of the pancreas^[4]. In the present case, such CT findings indicating malrotation were not immediately recognized due to the low frequency of this disease in the adult population. Malrotation was recognized only after a contrast enema showed that the entire colon was in the left half of the abdomen, with the cecum in the left lower quadrant. Furthermore, left-sided periappendiceal abscess was not suspected initially because there were no clinical characteristics suggesting appendicitis such as preceding vague central pain and there were only a few cases reported in the literature^[5].

We debated as to whether correction of the malrotation was indicated in this case. Dietz *et al*^[2] advocated that correction of the malrotation is probably not indicated unless there is evidence of intestinal obstruction. In contrast, Cathcart *et al*^[6] argued that surgical correction is warranted due to the risk of midgut volvulus.

In conclusion, all physicians with exclusively adult patients should be familiar with intestinal malrotation in order to make a timely and correct diagnosis that will lead to prompt and appropriate treatment.

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S- Editor Pan BR L- Editor Zhu LH E- Editor Bai SH

Stump appendicitis is a rare delayed complication of appendectomy: A case report

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Abstract

Stump appendicitis is an acute inflammation of the residual appendix and one of the rare complications after appendectomy. Paying attention to the possibility of stump appendicitis in patients with right lower abdominal pain after appendectomy can prevent the delay of diagnosis and treatment. In patients with stump appendicitis, CT scan not only assists in making an accurate preoperative diagnosis but also excludes other etiologies. We report a 47-year old man with preoperatively diagnosed stump appendicitis by CT, who underwent an open appendectomy 20 years ago.

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Key words: Stump appendicitis; Delayed complication; Preoperative diagnosis; Computed tomography; Incomplete appendectomy

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INTRODUCTION

Stump appendicitis is an acute inflammation of the residual appendix and a rare complication after appendectomy^[1]. Although the signs and symptoms do not differ from those of acute appendicitis, the diagnosis is often not considered because of prior appendectomy^[2]. A small number of stump appendicitis cases have been reported^[3]. We report a 47-year old man with preoperatively diagnosed stump appendicitis by CT, who underwent an open appendectomy 20 years ago.

CASE REPORT

A 47-year-old male patient presented to the emergency department with a chief complaint of abdominal pain for 6 d. The pain was initially localized around the umbilicus, and subsequently localized at the right lower quadrant. The patient complained of anorexia and nausea after the pain started. He had four or five episodes of watery diarrhea daily for 3 d though he had no vomiting. His surgical history included an appendectomy 20 years ago and surgery for peptic ulcer disease 15 years ago. His axillary temperature was 37.1°C, rectal temperature 38.4°C, blood pressure 125/70 mmHg, pulse rate 76 beats/min, and respiratory rate 16 breaths/min. Physical examination of the abdomen revealed healed midline and right lower abdominal (McBurney incision) surgical scars. A mass without clear borders was found showing tenderness, muscular guarding and rebound in the right lower quadrant during abdominal palpation. Routine laboratory tests included normal electrolyte and blood cell panels except for a white blood cell count of 11700/ μ L. Urinalysis was unremarkable. Abdominal ultrasonography (USG) showed a heterogeneous hypoechoic mass at the right lower quadrant. There was no abdominal free fluid. No specific diagnosis could be made. CT scan of the abdomen and pelvis showed a tubular structure extending from the cecum, with an enhanced wall consistent with an inflamed appendical stump (Figure 1A). Thickened cecum wall, inflammatory change of mesenteric fat and enlarged lymph nodes could also be seen (Figure 1B). Preoperative diagnosis of stump appendicitis was made on the basis of the CT findings.

Abdomen was opened with McBurney incision. Plastron formation in omentum and intestine at the right quadrant and inflammation at the cecum wall were seen. This site was dissected and the purulent material was drained. Stump appendicitis perforated from the appendix radix of 2 cm with necrosis was detected. Appendectomy was performed. The material with necrosis was removed and washed. A drain was placed and the abdomen was closed. Operative findings were confirmed by pathology. The patient was discharged from the hospital three days after operation.

DISCUSSION

Stump appendicitis is one of the rare delayed complications of appendectomy^[4]. Twenty-nine cases have been reported in the worldwide medical literature since the first case reported by Rose in 1945^[5,6]. We have detected 7 new

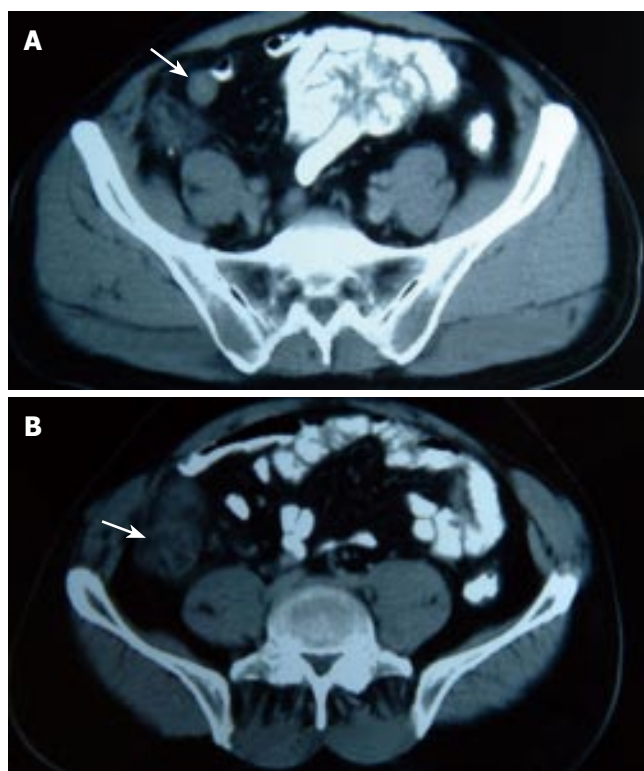


Figure 1 Contrast-enhanced CT scan of abdomen shows enlarged appendiceal stump with enhanced wall and local inflammatory changes (A) and distended cecum with thickening wall and stranding of adjacent mesenteric fat (B).

cases with our present case, thus 36 cases are available in the worldwide medical literature^[2,3,6-10].

The true incidence and prevalence of stump appendicitis are not known, but it is thought to be an underreported entity in the literature^[6]. The age of patients with stump appendicitis ranges from 11 to 72 years. The time intervals from initial appendectomy to stump appendicitis range from 2 mo to 51 years^[1,3]. A long appendiceal stump is reported to be a risk factor for stump appendicitis. The length of the stump ranges from 0.5 to 6.5 cm^[1,3,11,12]. Mangi and Berger^[12] reported that leaving an appendiceal stump less than 3 mm in depth with accurate visualisation of the appendiceal base, can minimise the incidence of stump appendicitis.

Whether simple ligation of the appendix or stump inversion into the cecum can reduce the risk of stump appendicitis remains controversial^[12]. Rao *et al*^[13] showed that all the cases reported in the literature undergo simple ligation of the appendix without invagination of the stump, suggesting that simple ligation with failure to amputate the appendix close to its origin from the cecum is a prerequisite for developing stump appendicitis. Mangi and Berger^[12] reviewed 2185 cases of appendectomy and found that there is no correlation between simple ligation and stump appendicitis. To leave the stump long may cause the inversion difficult. Therefore the length of stump may warn the surgeon.

Laparoscopic appendectomy is now a widely accepted surgical technique in treating appendicitis. The growing use of laparoscopic appendectomy may increase the frequency of stump appendicitis^[3,9,11,14] due to the potential

limitations of the technique such as smaller field of vision, lack of three-dimensional perspective, absence of tactile feedback, thus leaving a longer stump^[14]. In the reported 36 cases of stump appendicitis, only 10 had stump appendicitis after laparoscopic appendectomy^[15]. To date, no relationship between laparoscopic appendectomy and stump appendicitis has been demonstrated^[12].

A wide spectrum of diseases in the differential diagnosis of right lower quadrant pain of the abdomen and a past appendectomy history delay the diagnosis and treatment of stump appendicitis. It is usually diagnosed perioperatively when stump perforation and/or abscess occurs. In our case, the delay was due to the patient's late entry to the hospital.

Preoperative screening techniques may help to diagnose the disease^[2-4,13,16]. Only 1 case diagnosed by preoperative USG is available in the literature^[16]. In our case, although a pathologic lesion was detected at the right lower quadrant by USG, no specific finding of stump appendicitis was detected. Preoperative CT is a more efficient technique. To our knowledge, 4 cases have been diagnosed by CT having stump appendicitis apart from our case to date^[2-4,13]. In these studies, beside the appearance of inflamed appendix stump adjacent to the cecum, a cecal arrowhead sign indicating inflammation at the base of the appendix and appendicolith can be seen. CT findings may not be specific for stump appendicitis. Nonspecific inflammatory findings such as pericecal inflammatory changes, cecal wall thickening, abscess formation and fluid in the right paracolic gutter can be found in cases of stump appendicitis^[2-4,13].

In patients presenting with acute right lower quadrant findings mimicking appendicitis, the history of prior appendectomy does not absolutely rule out the possibility of appendicitis. But this situation usually causes an important delay of their diagnosis and treatment. In patients with stump appendicitis, CT scan not only assists in making an accurate preoperative diagnosis but also excludes other etiologies. Awareness of the possibility of stump appendicitis combined with a high index of suspicion can help to make an early diagnosis.

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CASE REPORT

Ruptured high flow gastric varices with an intratumoral arterioportal shunt treated with balloon-occluded retrograde transvenous obliteration during temporary balloon occlusion of a hepatic artery

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Abstract

A patient presented with hematemesis due to gastric variceal bleeding with an intratumoral arterioportal shunt. Contrast-enhanced CT revealed gastric varices and hepatocellular carcinoma with tumor thrombi in the right portal vein. Angiography and angio-CT revealed a marked intratumoral arterioportal shunt accompanied with reflux into the main portal vein and gastric varices. Balloon-occluded retrograde venography from the gastro-renal shunt showed no visualization of gastric varices due to rapid blood flow through the intratumoral arterioportal shunt. The hepatic artery was temporarily occluded with a balloon catheter to reduce the blood flow through the arterioportal shunt, and then concurrent balloon-occluded retrograde transvenous obliteration (BRTO) was achieved. Vital signs stabilized immediately thereafter, and contrast-enhanced CT revealed thrombosed gastric varices. Worsening of hepatic function was not recognized. BRTO combined with temporary occlusion of the hepatic artery is a feasible interventional procedure for ruptured high flow gastric varices with an intratumoral arterioportal shunt.

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Key words: Gastric varices; Hepatocellular carcinoma; Arterioportal shunt; Balloon-occluded retrograde obliteration; Hematemesis

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balloon-occluded retrograde transvenous obliteration during temporary balloon occlusion of a hepatic artery. *World J Gastroenterol* 2006; 12(33): 5404-5407

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INTRODUCTION

Hematemesis of ruptured high flow gastric varices from intratumoral arterioportal shunt is a critical condition. The transjugular intrahepatic portosystemic shunt (TIPS)^[1-2] and percutaneous transhepatic obliteration (PTO) are contraindications for portal hypertension accompanied with a marked intratumoral arterioportal shunt. Balloon-occluded retrograde obliteration (BRTO)^[3-8] also has never been reported as a treatment of high flow gastric varices with an intratumoral arterioportal shunt because stagnation of the sclerosing agent cannot be obtained. Endoscopic intravariceal sclerotherapy is applicable for the ruptured gastric varices. However, it might induce rebleeding at the puncture site as a complication.

We report a case in which BRTO combined with temporary balloon occlusion of a hepatic artery for bleeding gastric varices accompanied by the intratumoral arterioportal shunt was effective in thrombosing the varices resulting in a lifesaving outcome.

CASE REPORT

A 69-year old man with hepatocellular carcinoma (HCC) was referred to our hospital to undergo transcatheter arterial anticancer drug infusion and radiation therapy for tumor thrombi in the right portal vein visualized on contrast-enhanced CT. Angiography was performed to delineate the extent of the tumor thrombi and to infuse anticancer drugs from the proper hepatic artery. Celiac arteriography using a 4F catheter (RC2; Medikit, Tokyo, Japan) showed a hypervascular tumor stain in the right lobe and a marked intratumoral arterioportal shunt accompanied with reflux into the main portal vein and gastric varices (Figure 1). Then, the 4F catheter was advanced to the proper hepatic artery and computed

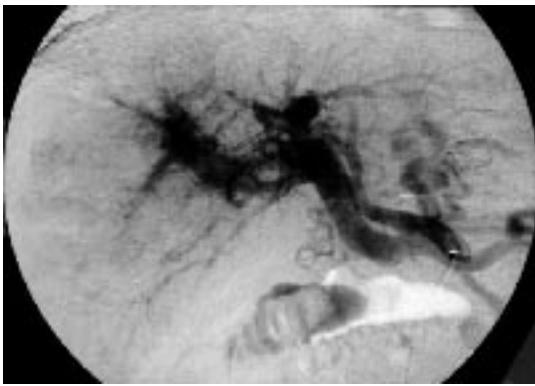


Figure 1 Celiac arteriography showing a marked arteriportal shunt with tumorous thread and streak sign and massive reverse flow to the main trunk of the portal vein.

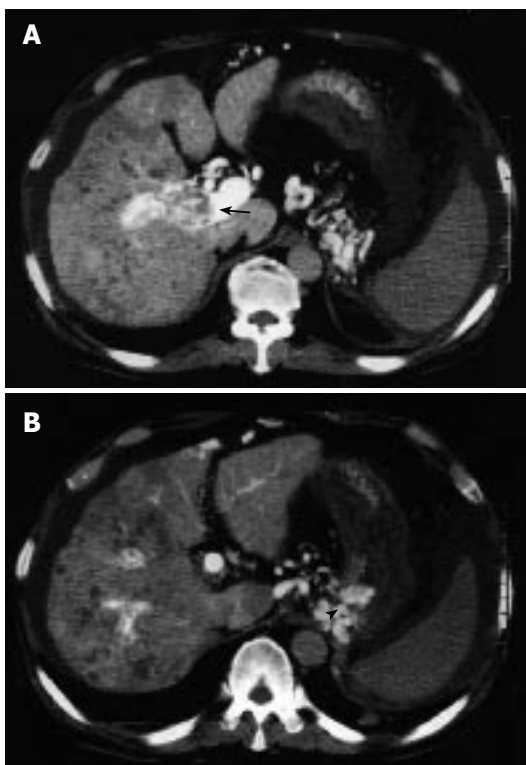


Figure 2 CT during proper hepatic arteriography showing tumor thrombi (arrow) in the right portal vein (A) and gastric varices (arrowhead) (B).

tomography during hepatic arteriography (CTHA) at 5 mm slices with injection of contrast agent (Iopamidol, 140 mgI; Daiichi, Tokyo) at a speed of 2 mL for a total volume of 60 mL. The CTHA showed HCC with the tumor thrombi in the right branch of the portal vein (Figure 2A) and a marked intratumoral arteriportal shunt with reflux into the main portal vein and gastric varices (Figure 2B). Anticancer drugs (10 mg mitomycin C and 40 mg farnorubicin) were infused from the proper hepatic artery. Ten days later, massive hematemesis occurred in the patient. Blood tests showed severe anemia and liver dysfunction (RBC/Hb/Hct 168/5.4/16.1, sGOT/sGPT/T.B 549/103/1.3). There was hesitation to perform an endoscopic intravariceal sclerotherapy because of apprehension of rebleeding by the direct puncture of the

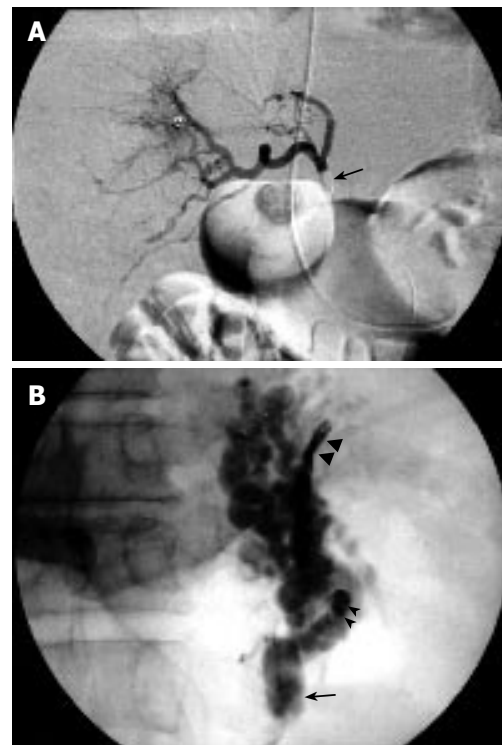


Figure 3 BRTO combined with temporary balloon occlusion of the hepatic artery. **A:** Proper hepatic arteriography through the balloon catheter during balloon occlusion (arrow); **B:** Balloon-occluded retrograde venography (arrow) after embolization of the left inferior phrenic vein and the retroperitoneal vein with micro-spring coils (arrowheads) showing the stagnant gastroduodenal shunt and gastric varices. A 5% ethanolamine oleate mixture (30 mL) was then injected during dual balloon occlusion.

high flow varices. Then, endovascular intervention was selected. First, a 14 mm diameter balloon catheter (Selecon 7F Straight MP catheter; Clinical Supply, Gifu, Japan) through a 7F sheath (J Sheath; Medikit, Tokyo, Japan) was inserted from the right jugular vein and advanced into the outflow pathway of a gastroduodenal (GR) shunt. The GR shunt was occluded by the inflated balloon and a retrograde injection of contrast agent was performed. However, the contrast agent was dispersed to the left inferior phrenic vein (IPV) and other flow tracts rapidly, and gastric varices were not visualized. Then, the left IPV and other flow tracts were embolized with micro-spring coils (Trufill; Cordis, Miami, FL and Tornade coil. Cook) through a 3 F microcatheter (Microphorette, Cook). After embolization of their tracts, however, the gastric varices remained unopacified by balloon-occluded retrograde venography. High blood flow through the arteriportal shunt was the main cause. In the absence of stagnation of the contrast agent, complete thrombolization of the gastric varices was impossible by BRTO and the complications due to massive systemic leakage of ethanolamine oleate (EO) as a sclerosing agent were also feared. In order to stagnate the blood flow of GR shunt, temporary balloon occlusion of the proper hepatic artery was performed using a 9 mm balloon catheter (Selecon, 5F Straight, Clinical Supply) (Figure 3A). Under temporary balloon occlusion of the proper hepatic artery, the outflow pathway of the GR shunt was reclosed by inflating the 14 mm diameter balloon. It led to the visualization

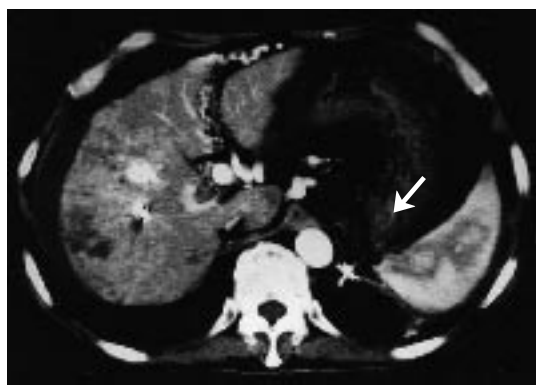


Figure 4 Contrast-enhanced CT showing thrombosed gastric varices (white arrow) on the following day.

of the stagnant gastric varices with balloon-occluded retrograde venography. Five percent EO solution, which is a mixture of 10% EO (Oldamin; Grelan Pharmaceutical, Tokyo, Japan) and the same dose of a nonionic contrast medium (Iopamiron 370 mgI/dL; Schering Osaka, Japan), were slowly injected into the GR shunt during the dual balloon occlusion after venous drip infusion of human haptoglobin (Mitsubishi Pharma, Osaka, Japan) at a total volume of 4000 units. The gastric varices were filled with 30 mL of 5% EO solution (Figure 3B). The balloon catheter in the proper hepatic artery was deflated and removed after one hour. Patency of the proper hepatic artery was confirmed by contrast agent injection from the catheter before removal. The balloon occlusion of the GR shunt was maintained for 24 h. Contrast-enhanced CT performed on the following day showed thrombosed gastric varices (Figure 4). Following BRTO, blood pressure and other vital signs stabilized, and progression of anemia was halted. Worsening of hepatic function was not recognized (sGOT/sGPT/T.B 108/73/1.3). After that, the patient could undergo radiation therapy for the portal tumor thrombi.

DISCUSSION

Kanagawa *et al*^[3] first introduced BRTO as an effective therapy for gastric varices in 1990. BRTO is performed by injecting a sclerosing agent from the balloon catheter through the outflow pathway of the gastrorenal shunt during balloon occlusion^[3,4]. The success rate of gastric varix sclerosis reaches 85%-100%^[3-8]. Five percent ethanolamine oleate iopamidol (EOI) is usually used as the sclerosing agent with retrograde injection. Massive systemic leakage of ethanolamine oleate causes various complications such as acute respiratory distress syndrome^[9,10], acute renal disorder^[11] and disseminated intravascular coagulation^[12]. Though human haptoglobin^[11,13] has been used to prevent such complications, leakage into the systemic circulation must be minimized. In cases of multiple outflow tracts such as inferior phrenic vein, pericardiophrenic vein, and other outflows, embolization of these outflow tracts with micro-spring coils is performed to reduce the injection volume of EO and prevent systemic leakage^[8].

In our case, BRTO alone was anticipated to lead to inadequate occlusion of the outflow tract and massive systemic leakage of EOI because of high blood flow from the intratumoral arteriportal shunt. According to the classification of the degree of progression of gastric varices^[6], the high flow gastric varices in which the GR shunt cannot be occluded with the balloon catheter alone, belong to the highest grade 5. In patients with the most progressive gastric varices, partial splenic embolization and PTO of the coronary vein as a main inflow route are required to reduce the blood flow into varices before BRTO^[6]. However, no description about the procedure to treat gastric varices accompanied with a marked intratumoral arteriportal shunt is available. Transcatheter embolization is an effective treatment for variceal bleeding due to portal hypertension caused by arteriportal fistulae^[14-16]. In cases of portal tumor thrombi, however, transcatheter embolization of the hepatic artery with gelatin sponge particles, metallic coils and other permanent embolic materials to reduce the arterial flow into varices can cause a large liver infarction and hepatic failure^[17]. Temporary occlusion of the proper hepatic artery using a balloon catheter to reduce blood flow through the arteriportal shunt prevents liver infarction, minimizes systemic leakage of EO and permits complete thrombosis of the gastric varices by concurrent BRTO. Temporary balloon-occluded proper hepatic arterial infusion of anticancer drugs can be performed for HCC with portal tumor thrombi and is a safe procedure because short-term ischemia has little effect on hepatic function^[18]. In this case, there was no deterioration of liver function after BRTO during temporary balloon occlusion of the hepatic artery. There is no previous report describing the feasibility of BRTO during temporary balloon occlusion of the hepatic artery for hemorrhagic gastric varices with a marked intratumoral arteriportal shunt.

In conclusion, BRTO combined with temporary occlusion of a proper hepatic artery is a safe and useful method for the treatment of ruptured high flow gastric varices with a marked intratumoral arteriportal shunt.

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CASE REPORT

Successful treatment of giant rectal varices by modified percutaneous transhepatic obliteration with sclerosant: Report of a case

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Abstract

We present a female patient with continuous melena, diagnosed with rectal variceal bleeding. She had a history of esophageal varices, which were treated with endoscopic therapy. Five years after the treatment of esophageal varices, continuous melena occurred. Since colonoscopy showed that the melena was caused by giant rectal varices, we thought that they were not suitable to receive endoscopic treatment. We chose the modified percutaneous transhepatic obliteration with sclerosant, which is one of the interventional radiology techniques but a new clinical procedure for rectal varices. After the patient received this therapy, her condition of rectal varices was markedly improved.

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Key words: Rectal varices; Modified percutaneous transhepatic obliteration with sclerosant

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INTRODUCTION

Ectopic varices outside the esophagogastric lesion are rare in patients with portal hypertension^[1]. Among ectopic varices, rectal varices are comparatively common, but their rupture is often fatal although it is rarely reported^[2]. Though there are several case reports of rectal varices treated with endoscopic variceal ligation (EVL)^[3], endoscopic injection sclerotherapy (EIS)^[4,5], transjugular intrahepatic portosystemic shunt (TIPS)^[6,7] and surgery^[8,9], no single effective method has yet been established. Kimura *et al*^[10] have reported that a patient is successfully treated with a new interventional radiological procedure, double balloon-occluded embolotherapy (DBOE). We used the modified percutaneous transhepatic obliteration with sclerosant (MPTO) rather than DBOE in the treatment of giant rectal varices in our case.

CASE REPORT

A 77-year old woman with liver cirrhosis had bleeding from esophageal varices, which was treated with EIS in 1992. She presented with profuse melena in March 1998 and was admitted to a nearby hospital due to shock. She received 2000 mL of packed red blood cells and was transferred to our hospital for further treatment. On admission, physical examination revealed moderate anemic conjunctiva without scleral icterus. Tense ascites was present without abdominal tenderness. The functional reserve of the liver was assessed as Child-Pugh grade B. Esophago- gastroduodenoscopy did not find recurrence of gastroesophageal varices. Flexible sigmoidoscopy revealed huge tortuous rectal varices extending from outside the anus to the recto-sigmoid junction (Figure 1). Endoscopic Doppler ultrasonography (FG-32UA, Pentax Co., Ltd., EUB-555 US scanner, Hitachi Co., Ltd., Tokyo Japan) revealed that the largest diameter of varices was 8.8 mm and the blood flow velocity was 5 cm/s. Percutaneous transhepatic inferior mesenteric venography and pelvic angiography revealed that the blood of the rectal varices was supplied by the inferior mesenteric vein and flowed into the bilateral internal iliac vein *via* the internal pudendal vein (Figure 2). Percutaneous transhepatic portography revealed narrowing of the portal vein trunk

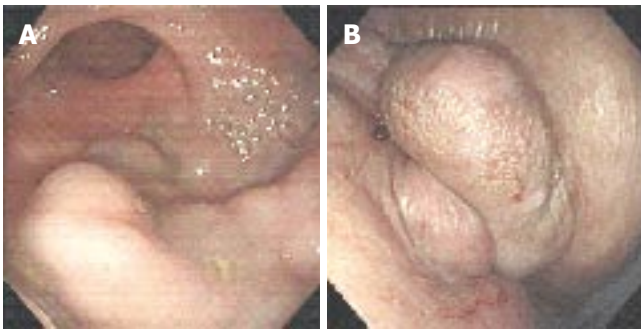


Figure 1 Flexible sigmoidoscopy reveals huge tortuous rectal varices extending from outside the anus to the recto-sigmoid junction (A: rectum, B: outside the anus).



Figure 2 Percutaneous transhepatic portography demonstrates that the rectal varices are supplied by the inferior mesenteric vein (arrow) and flowed into the bilateral internal iliac vein (arrowhead).

and thrombosis of the superior mesenteric vein. For such extremely large rectal varices, treatment with EIS or EVL did not seem feasible. Instead, it was decided to treat her rectal varices with MPTO.

An occlusion balloon catheter (maximum external diameter of the balloon was 20 mm) and metallic coil were placed into the inferior mesenteric vein through the percutaneous transhepatic portogram route. When the rectal varices were fully visualized and contrast medium did not flow into the internal iliac vein, 5 mL of absolute ethanol was injected through the occluded inferior mesenteric vein followed by 7 mL of 5% ethanolamine oleate iopamidol (EOI) (Figure 3). The balloon was kept inflated for about 24 h. Twenty-four hours after the embolotherapy, PTP detected no blood flow in the rectal varices. There were no major complications during hospitalization. Four weeks after the embolotherapy, the sizes of the varices became markedly smaller (Figure 4). No rectal variceal bleeding occurred after treatment.

DISCUSSION

Rectal varices result from dilated submucosal veins which extend from the dentate line into the rectum and represent the portosystemic collaterals between the superior rectal vein flowing into the inferior mesenteric vein, and the

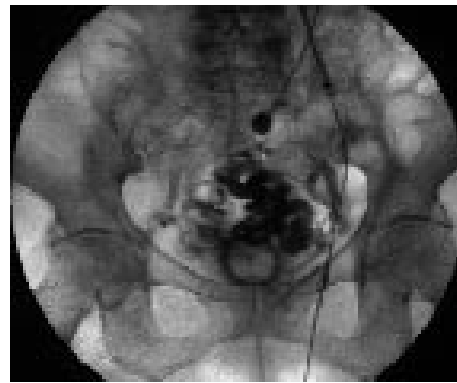


Figure 3 The sclerosant stagnates in the varices as a result of occlusion of the blood flow of the inferior mesenteric vein.



Figure 4 Sigmoidoscopy reveals disappearance of huge tortuous rectal varices extending from outside the anus to the recto-sigmoid junction (A: rectum; B: outside the anus).

middle and inferior rectal veins draining into the internal iliac vein^[11,12]. They are thought to be distinct from hemorrhoids, which do not occur in the rectum and have no direct communication with the portal circulation^[12-14].

The first case of rectal varices was reported in 1954^[15]. Izsak and Finlay^[16] have reported 29 cases and Gudjonsson *et al*^[17] have reviewed 69 cases of colonic varices since then. The incidence of colonic varices is low and has been reported to be 0.07%^[18].

The most common underlying condition associated with rectal varices is portal hypertension, which is present in approximately three-fourths of all cases^[17]. Other causes include localized portal hypertension resulting from blood flow disturbances through the mesenteric veins as a result of postoperative adhesion^[19], splenic vein obstruction associated with pancreatitis^[20], and carcinoid-induced mesenteric vein obstruction^[21]. In addition, familial vascular anomalies^[22], congenital vascular anomalies^[23], and congestive heart failure^[17] have been suggested as the possible causes of rectal varices. In our case, the patient received esophageal sclerotherapy six years ago. Keane *et al*^[24] have reported a case of massive bleeding from rectal varices following repeated injection sclerotherapy for esophageal varices. An incidental risk of rectal varices after eradication of esophageal varices seems to be increased where portal hypertension is present.

Previously reported therapies for rectal varices include EIS^[4,5], EVL^[3], TIPS^[6,7], surgery^[8], and more conservative

therapies such as drip infusion of vasopressin^[25]. There is no standardized treatment for rectal varices, but rectal varices are accessible through standard endoscopy. Several studies have reported their successful treatment with sclerosant injection or band ligation. Endoscopic therapy with sclerosant is not so satisfactory, especially for large rectal varices, because sclerosant may be diluted in large varices beyond a concentration sufficient to obliterate the varices and there may be a risk of developing severe complications, such as pulmonary embolism, due to the flow of high doses of sclerosant into the systemic circulation. EVL can be used to treat active bleeding from rectal varices as an emergency treatment modality. Norton *et al*^[26] reported that if the entire varix cannot be banded there is a risk of causing a wide defect in the varix after sloughing of the band, rendering the banding technique unsafe for large rectal varices.

Since surgery imposes a substantial burden and risk on patients, it may be advisable to reserve it for patients with Child-Pugh class A and extrahepatic portal vein thrombosis as the cause of portal hypertension. The efficacy of mobilization therapy or portosystemic shunt therapy using radiological techniques in the treatment of bleeding ectopic varices is rarely reported^[27]. There are some reports on successful treatment of severe rectal varices with TIPS^[6,7]. However, other reports showed that patients may die of rapidly progressive hepatic failure after TIPS procedure^[28]. We have only used it in the treatment of patients on a waiting list for liver transplantation. Only one report on embolization therapy for rectal varices is available in 1900 and 2005. Kimura *et al*^[10] reported that double balloon-occluded embolotherapy (DBOE) can lead to obliteration rectal varices. However, they have not stated whether contrast medium is stagnated in the varices as a result of occlusion of blood flow in the inferior mesenteric vein. In our case, since percutaneous transhepatic inferior mesenteric venography revealed that the blood of rectal varices was supplied by the inferior mesenteric vein only, we chose MPOT to treat the rectal varices, as it is an easier and less invasive procedure than DBOE. The total amount of sclerosant needed for MPOT or DBOE was 12 mL. If contrast medium stagnates in the rectal varices due to occlusion of the inferior mesenteric vein, as in our case, MPOT may be appropriate.

MPOT can also result in complications, such as renal failure causing hemoglobinuria because of hemolysis, and those associated with percutaneous transhepatic catheterization. Ohta *et al*^[29] reported that intraperitoneal bleeding after retraction of the catheter is found in 10.6% patients with rectal varices. These complications can also be seen after DBOE. MPOT can close the in-flow and out-flow of rectal varices by occluding the blood flow of the inferior mesenteric vein.

In conclusion, MPOT with sclerosant may prove to be an extremely useful method in the treatment of rectal varices. Further studies are needed to evaluate the efficacy and safety of this method.

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CASE REPORT

An unusual cause of hematemesis: Goiter

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Abstract

Downhill varices are located in the upper part of the esophagus and are usually related to superior vena cava obstruction. Bleeding from these varices is extremely rare. We describe a 77-year-old patient with hematemesis due to downhill varices as a result of recurrent goiter. A right lobe thyroidectomy was carried out with disappearance of the varices.

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Key words: Downhill; Esophageal; Varices; Goiter; Hematemesis

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INTRODUCTION

Bleeding from distal esophageal varices is a frequent complication of portal hypertension. The portal blood drains into the superior vena cava by gastric and esophageal collaterals, also called uphill varices, referring to the upward direction of blood flow to the superior vena cava. In contrast to uphill varices, downhill varices have a retrograde blood flow and are located in the proximal esophagus. Downhill varices are rare and usually caused by superior vena cava obstruction due to bronchogenic carcinoma and mediastinal tumors, *etc*^[1,2]. They serve as collaterals either to bypass superior vena cava obstruction via the azygos vein or to drain the superior system to the

portal vein when both the superior vena cava and the azygos vein are occluded. In contrast to the high risk of hemorrhage from uphill varices in portal hypertension, bleeding from downhill varices is extremely rare. Although an increased variceal wall tension is the ultimate factor causing bleeding in both types of varices, several factors may underlie this difference in bleeding tendency. First, in patients with uphill varices, coagulation capacity may be reduced due to concomitant liver disease with an inherently increased bleeding tendency. Second, exposure to esophagogastric reflux damages distal rather than proximal varices. Third, because distal uphill varices predominantly distend at subepithelial levels compared to the submucosal location of downhill varices in the midthoracic and proximal esophageal wall, variceal rupture is much more likely to occur near the esophagogastric junction^[2]. We report a rare case of bleeding downhill varices in the absence of superior vena cava obstruction. Detailed diagnostic work-up showed that the downhill varices were caused by goiter. The varices disappeared after subtotal thyroidectomy.

CASE REPORT

A 77-year-old female was admitted to the hospital because of hematemesis. The patient had a one-year history of dysphagia and weight loss. The patient had recurrent goiter after a subtotal thyroidectomy in 1979 for multinodular goiter. Her medical history also revealed chronic obstructive pulmonary disease (COPD) and smoking. The medication consisted of inhalation of salbutamol and ipratropium bromide. Physical examination revealed a pale non-icteric woman with normal vital signs and a normal voice without stridor. There was a large, firm, nodular mass on the right side of the neck with a horizontal thyroidectomy scar. There were no dilated veins and no bruits audible over the mass. Physical signs of liver disease, Graves' disease and superior vena cava syndrome were absent.

Laboratory findings including thyroid function tests were normal except for 7 mmol/L hemoglobin (normal: 7.5-10.0 mmol/L) and 0.31% hematocrit (normal: 0.37%-0.47%), respectively. At emergency gastroscopy, the descending part of the duodenum and duodenal bulb were unremarkable. The stomach showed no abnormality except for a few adherent streaks of blood and a small amount of dark blood without clots. Careful gastric mucosal examination after use of a water jet was again unremarkable. The distal esophagus appeared normal

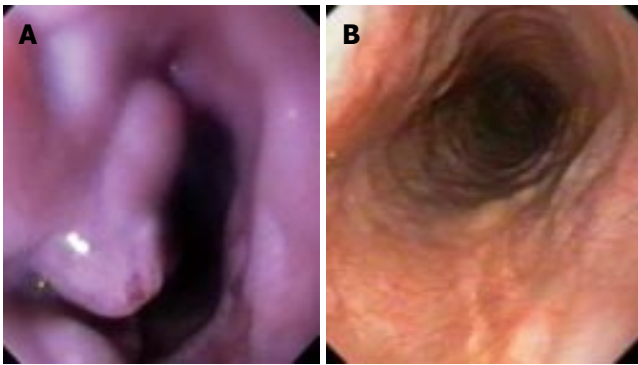


Figure 1 Endoscopic view demonstrating downhill varices (A) and almost complete disappearance of the downhill varices (B) before and 12 mo after thyroidectomy of the right lobe.

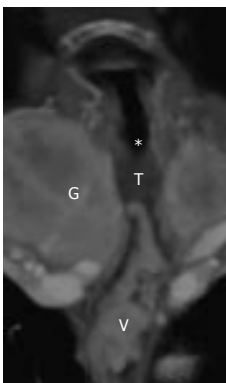


Figure 2 3D reconstruction of CT angiography with view at the dorsal wall of the trachea (T) demonstrating a venous plexus of downhill varices (V) on the wall between the esophagus and trachea connected with a thyroid vein at the goiter (G) of the right thyroid lobe; *, out of plane, cut off level thick slice.

without signs of reflux esophagitis or Mallory-Weiss lesion. However, proximal grade II–III downhill esophageal varices were visualized 6 centimeters below the upper esophageal sphincter. Importantly, one of the varices showed a small fibrin plug indicating recent bleeding. Since bleeding stopped spontaneously, no treatment was given except for blood transfusion and a diet of semi-solid food. A second gastroscopy after 48 h showed again grade II–III varices without signs of (re)bleeding (Figure 1A). CT of the thorax showed a substernal goiter and a slight compression of the trachea without any other abnormalities. CT angiography demonstrated a dilated venous plexus around the esophagus connected with a thyroid vein (Figure 2, Figure 3A). The venous plexus extended over 7.4 centimeters from the seventh cervical vertebra to the third thoracic vertebra and drained into the azygos vein. There was compression of the right internal jugular vein near the junction of the brachiocephalic vein and a dilatation of the more cranial part. Ultrasound and Doppler examination demonstrated a craniocaudal blood flow. Flebography excluded superior vena cava obstruction. Arteriography of the aortic arch excluded the presence of arteriovenous malformations. Since goiter seemed to cause the downhill varices, a thyroidectomy of the right lobe was carried out sparing the right recurrent laryngeal nerve and both parathyroids. Intraoperatively, a dilated right internal jugular vein was seen. The thyroid specimen weighed 85 g and was histopathologically diagnosed as multi-nodular goiter. The patient had a satisfactory

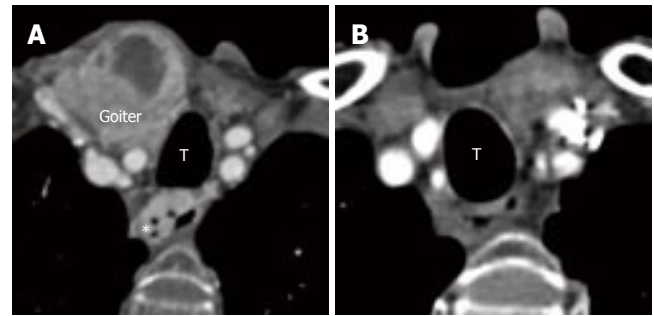


Figure 3 CT angiography demonstrating downhill varices one day after hemorrhage (A) and disappearance of the downhill varices 12 mo after thyroidectomy of the right lobe (B). * indicates varices around the esophagus; T, trachea.

recovery and was euthyroid after surgery with dysphagia complaints improved during follow-up. Twelve months after surgery, endoscopy and CT angiography showed almost complete disappearance of the varices (Figures 1B, 3B). Twenty months postoperation the patient was still non-symptomatic.

DISCUSSION

Downhill varices are usually associated with superior vena cava obstruction due to bronchogenic carcinoma, different types of mediastinal tumor and fibrosis, venulitis, surgical caval ligation and thyroid masses^[1,2]. Occasionally, as in our patient, downhill varices may develop without superior vena cava obstruction^[3,4]. Relatively few case reports have been published on bleeding downhill varices in relation to thyroid pathology (Table 1). In a study of 1051 patients with cervical and retrosternal goiter, 3% of patients developed non-bleeding downhill varices^[13]. Lagemann^[14] performed barium swallows in 50 patients with recurrent thyroid enlargement and demonstrated that more than 50% of the patients have non-bleeding downhill varices. Blood from the thyroid plexus flows through the inferior thyroid veins (also called thyroid ima veins) into the brachiocephalic vein. In case of obstruction of the inferior thyroid veins, blood flows *via* the deep esophageal veins leading to esophageal varices. The esophageal varices can drain into collaterals to the brachiocephalic, azygos, hemiazygos and accessory hemiazygos veins, all of which finally drain into the superior vena cava. In the present case, goiter caused compression of the internal jugular vein. Blood flow over the thyroid plexus draining into the inferior thyroid veins might bypass compression of the internal jugular vein. However, in this patient downhill varices developed and bypassed this compression, suggesting that the function of inferior thyroid veins is insufficient. Both previous thyroidectomy and recurrent goiter are possible explanations, since inferior thyroid veins can be occluded either by primary or recurrent thyroid tumors or by surgical ligation during thyroidectomy and fibrogenesis or mediastinitis secondary to surgery. In the present case, the downhill varices drained into the azygos vein as illustrated in Figure 4. Hemorrhage of downhill varices is an emergency. However, the experience with treatment is limited because of its rare bleeding propensity.

Table 1 Case reports on bleeding downhill varices associated with thyroid pathology

Year	Author	Aetiology	SVCO	Treatment	Outcome
1960	Sundermann and Kämmerer ^[5]	Recurrent goiter	No	Thyroidectomy	Barium swallow after six years: disappearance of varices
1976	Barber <i>et al</i> ^[6]	Goiter	No	Thyroidectomy	Endoscopy after four months: disappearance of varices
1978	Johnson <i>et al</i> ^[7]	Carcinoma	Yes	Thyroidectomy	Barium swallow after two months: disappearance of varices
1982	Fleig <i>et al</i> ^[8]	Recurrent goiter	No	Sengstaken-Blakemore tube	Endoscopy after two weeks: still varices
1982	Kelly <i>et al</i> ^[9]	Goiter	No	Thyroidectomy	Venogram after one month: disappearance of varices
1986	Takahashi <i>et al</i> ^[10]	Recurrent goiter	No	Thyroidectomy	Thyroid arteriography after three weeks: disappearance of varices
1998	Tsokos <i>et al</i> ^[11]	Recurrent goiter	No	Sclerotherapy	Death caused by pulmonary embolism of cyanoacrylate used for sclerotherapy Endoscopy and venography after 4 mo:
2006	Bédard and Deslauriers ^[12]	Posterior mediastinal goiter	Yes	Resection of mediastinal mass	disappearance of varices and SVCO syndrome
2006	van der Veldt <i>et al</i> current report	Recurrent goiter	No	Thyroidectomy	Endoscopy and CT angiography after twelve months: almost complete disappearance of varices

SVCO: Superior vena cava obstruction.

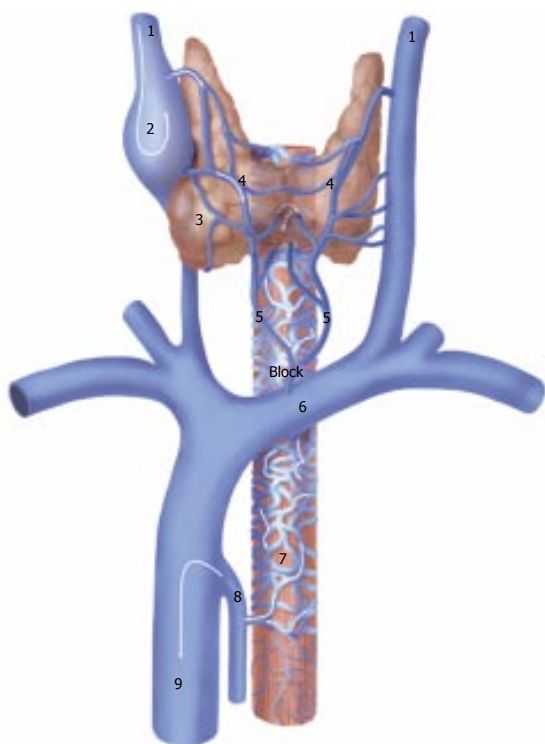


Figure 4 Venous blood flow of the downhill varices in relation to goiter. 1: internal jugular vein; 2: dilated right internal jugular vein; 3: goiter with compression of the right internal jugular vein; 4: thyroid plexus draining into the esophageal varices; 5: inferior thyroid vein; 6: brachiocephalic vein; 7: varices around the esophagus; 8: azygos vein; 9: superior vena cava; Block: Occlusion of the inferior thyroid vein, possibly as a result of previous surgery or recurrent goiter.

In contrast to uphill varices, endoscopic sclerotherapy is not generally performed to treat downhill varices up to 5 cm below the upper esophageal sphincter, because retrograde flow of sclerosant through the azygos vein

could result in spinal cord and vertebral body infarction^[15]. Fatal pulmonary embolism of cyanoacrylate used for endoscopic embolization of downhill varices has also been reported^[11]. Therefore, downhill varices should be recognized and distinguished from uphill varices. The use of a Sengstaken-Blakemore tube can be lifesaving^[8]. Endoscopic band ligation has been shown to be effective in preventing recurrent bleeding of downhill varices^[2]. Finally, definitive treatment is performed to eliminate the cause of venous obstruction. As in this patient, surgery can successfully relieve obstruction. For goiter-related downhill varices jodium therapy can also be effective^[8].

In conclusion, downhill varices although rare, can cause upper gastrointestinal bleeding and should be suspected in any patient with evidence of thyroid enlargement or having a history of thyroid surgery, even though signs of superior vena cava obstruction are absent. Management of the underlying cause, as in this case by thyroidectomy, can efficiently lead to recovery and disappearance of the esophageal varices.

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CASE REPORT

Hepatic venous outflow obstruction after piggyback liver transplantation by an unusual mechanism: Report of a case

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Abstract

Hepatic venous outflow obstruction after piggyback liver transplantation is a very rare complication. An unusual mechanism aggravating it is reported. A 33-year-old man with end-stage hepatitis B liver cirrhosis underwent a piggyback orthotopic liver transplantation using a full-size cadaveric graft. Two months after transplantation, he developed gross ascites refractory to maximal diuretic therapy. Doppler ultrasound showed patent portal and hepatic veins. Serial computed tomography scans revealed a hypoperfused right posterior segment of the liver which subsequently underwent atrophy. Hepatic venography demonstrated a high-grade stenosis with an element of torsion of venous drainage at the anastomosis. The stenosis was successfully treated with repeated percutaneous balloon angioplasty. The patient remained asymptomatic six months afterwards with complete resolution of ascites and peripheral edema. We postulate that liver allograft segmental hypoperfusion and atrophy may aggravate or result in a hepatic venous outflow problem by the mechanism of torsion effect. Percutaneous balloon angioplasty is a safe and effective treatment modality for anastomotic stenosis.

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Key words: Hepatic venous outflow obstruction; Piggyback; Liver transplantation; Percutaneous balloon angioplasty

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INTRODUCTION

Hepatic venous outflow obstruction after piggyback

orthotopic liver transplantation is a rare complication^[1,2]. However, failure of early recognition and treatment of this complication can result in graft failure and even death of patients. We report here a case of hepatic venous outflow obstruction after piggyback orthotopic liver transplantation aggravated by an unusual mechanism and its successful treatment with percutaneous balloon angioplasty.

CASE REPORT

A 33-year-old man underwent cadaveric orthotopic liver transplantation for end-stage hepatitis B liver cirrhosis. A full-size graft was used and the caval anastomosis was performed with piggyback technique. Two weeks after transplantation, he was noted to have persistent leukocytosis ($27-37 \times 10^9/L$) though he was afebrile. A computed tomography (CT) scan of the abdomen was arranged to rule out intraabdominal collection or abscess formation. It revealed an extensive wedge-shaped region of low attenuation and decreased perfusion involving the right posterior segment of the liver graft, suggestive of an evolving infarct (Figure 1). He was treated conservatively with antibiotics and his white blood cell count gradually returned to a normal level.

Six weeks later, the patient developed rapidly progressive abdominal distension and edema in the lower limbs. Ultrasound of the abdomen showed gross ascites, but the portal and hepatic veins were patent. The ascites failed to respond to maximal diuretic therapy and paracentesis. He subsequently underwent a venography which demonstrated a high-grade stenosis with an element of torsion of venous drainage at the anastomosis (pressure gradient 33 mmHg) (Figures 2 and 3). A repeated CT scan showed that the right posterior segment (previously hypoperfused segment) underwent atrophy (Figure 4). The venous outflow obstruction was treated with repeated percutaneous balloon angioplasty with a 10 mm and a 14 mm balloons (Figure 5A, 5B). Pressure gradient across the venous anastomosis was successfully reduced from 33 mmHg to 2 mmHg. The patient remained asymptomatic six months afterwards with complete resolution of ascites and peripheral edema.

DISCUSSION

Standard orthotopic liver transplantation, as described by Starzl *et al*^[3] in 1963, involves an anhepatic phase during which the retrohepatic vena cava is resected. The main drawback of this classical technique is the decrease in venous return to the heart during this anhepatic



Figure 1 Computed tomography scan showing hypoperfusion at the right posterior segment of the liver graft.

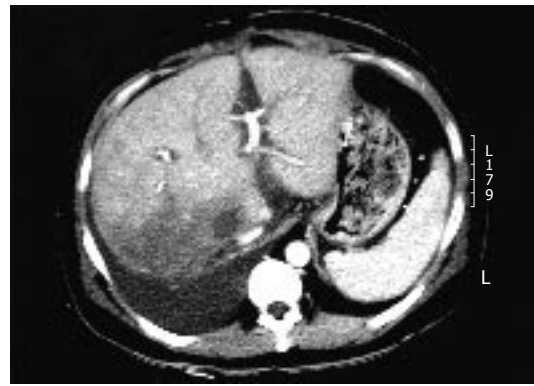


Figure 4 Computed tomography scan showing atrophy of the right posterior segment of the liver graft.



Figure 2 Hepatic veins are not opacified on inferior venacavogram, suggestive of the presence of stenosis at the caval anastomosis.

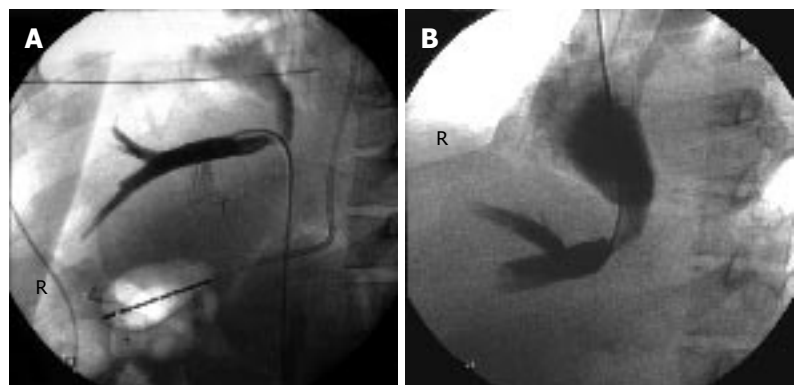


Figure 5 Post-angioplasty (with 10 mm balloon) hepatic venogram showing improvement of the stenosis (A) and the stenotic segment to almost normal caliber (B).



Figure 3 Selective hepatic venogram showing high-grade stenosis at the caval anastomosis.

phase, which causes haemodynamic instability and renal impairment. The introduction of the extracorporeal venovenous shunt has circumvented these physiological complications from occurring. In late 1960s, an alternative technique with vena cava preservation was described by Sir Roy Calne *et al*^[4]. This technique was modified by Tzakis *et al*^[5] in 1989 and has become the well known piggyback technique since then. It has the advantages of preservation of the vena caval blood flow and maintenance of venous

return to the heart in the anhepatic phase. Haemodynamic alterations are avoided without the need of venovenous bypass. Many transplant centres are now favouring the piggyback technique as the technique of choice in both paediatric and adult liver transplantation.

Hepatic venous outflow obstruction from anastomotic stricture is a very rare complication after piggyback orthotopic liver transplantation, particularly in adult recipients who receive full-size liver grafts. In a retrospective study of 1112 piggyback liver transplantations performed in seven transplant units in Spain, the reported incidence of hepatic venous outflow obstruction is about 1%^[1]. In another review of 264 piggyback transplantations performed at Stanford University Medical Center, only two patients were found to have developed venous outflow obstruction, representing an incidence of 0.8%^[2]. The causes of hepatic venous outflow obstruction after piggyback liver transplantation are generally believed to be due to either anastomotic discrepancy or kinking of the venous anastomosis^[6,7]. Paediatric recipients receiving reduced-size liver grafts are more prone to develop this complication because of size discrepancy between the donor suprahepatic vena cava and the recipient vein cuff. The presence of a wide empty subphrenic space and the lack of peritoneal attachments are other possible factors that permit a small liver graft to rotate around the venous anastomosis axis and hence lead to anastomotic kinking

and obstruction. In adult liver transplantation, graft torsion is less likely to happen if a full-size graft is used because this effect is virtually eliminated by the presence of both liver lobes.

In our patient, a full-size liver graft was used, and theoretically the risk of graft torsion and venous anastomotic kinking should be very low. However, this rare complication of hepatic venous outflow obstruction still arose. On serial CT scans of the abdomen after transplantation, the right posterior segment of the liver graft was shown to be ischaemic and hypoperfused initially. The subsequent atrophy of the same segment resulted in rotation which in turn led to the kinking of the venous anastomosis. This unusual mechanism of hepatic venous outflow obstruction after piggyback liver transplantation, to the best of our knowledge, has never been described in the literature before.

Patients with hepatic venous outflow obstruction usually present with massive ascites and bilateral lower limb edema between 2 and 16 mo post-transplantation, which is refractory to oral protein supplements and maximal diuretic therapy^[1,2]. Some of the patients can develop acute Budd-Chiari syndrome early within the first week of post-transplantation^[1]. Failure of recognition of this complication can result in graft failure and death of patients. Diagnosis must be confirmed by hepatic venography and a pressure gradient study. Emergency revision of anastomosis or even retransplantation is occasionally required to remedy the condition. Nowadays with the advancement of minimally invasive endovascular intervention, selected cases of hepatic venous outflow obstruction can be successfully treated with percutaneous balloon angioplasty and stenting^[2,8,9]. The procedure is done under local anaesthesia with minimal morbidity and mortality. Symptoms of ascites and peripheral edema are relieved rapidly and dramatically after the treatment. Repeated balloon angioplasty is sometimes needed to treat recurrent stenosis. The combination of balloon angioplasty and stenting can improve the long-term durability of the treatment.

In conclusion, liver allograft segmental hypoperfusion and atrophy may aggravate or result in hepatic venous outflow obstruction by the mechanism of torsion effect. Hepatic venous outflow obstruction should be considered in all patients who present with intractable ascites after liver transplantation. Venography is mandatory for diagnosis. Percutaneous balloon angioplasty is a safe and effective treatment modality for anastomotic stenosis.

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Primary squamous cell carcinoma of the liver: A successful surgically treated case

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Abstract

Primary squamous cell carcinoma (SCC) of the liver is rare. Totally nine such cases have been reported in the literature. Primary SCC of the liver has been reported to be associated with hepatic teratoma, hepatic cyst, or hepatolithiasis. Complete remission of poorly differentiated SCC of the liver could be achieved by systemic chemotherapy followed by surgery or remarkably respond to hepatic arterial injection of low dose chemotherapeutic drugs. Here we report the first case of primary SCC of the liver presenting as a solid tumor and receiving successful hepatic resection with 9-mo disease free survival.

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Key words: Squamous cell carcinoma; Liver; Surgery

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INTRODUCTION

Primary squamous cell carcinoma (SCC) of the liver is rare and reported sporadically. It has been reported to be associated with hepatic teratoma, hepatic cysts, or hepatolithiasis^[1-3]. Boscolo showed that a case of complete remission of poorly differentiated SCC of the liver could be achieved by systemic chemotherapy followed by surgery^[4]. Kaji demonstrated that a case of primary SCC of the liver could remarkably respond to hepatic arterial injection of low dose chemotherapeutic drugs^[5].

To our knowledge, a total of nine cases of primary SCC of the liver have been reported and here we report a case presenting as a solid tumor and receiving successful hepatic resection with disease free survival for 9 mo^[1-9].

CASE REPORT

A 40-year-old man presented with right upper quadrant (RUQ) pain for several weeks before admission in September 2005. He did not drink alcohol, smoke, or have hepatitis or a history of surgery. On admission, he had a fever and temperature elevated to 38°C. There was no vomiting, jaundice, dysuria, chills, or abdominal distention. Physical examination revealed tenderness over the RUQ area without a palpable abdominal mass. The following laboratory data were recorded: hemoglobin 13.5 g/dL; white blood cell count 14 600/mm³; platelet: 421 000/μL; prothrombin time (test/normal control): 13.4/11.2 s; international normalized ratio (INR): 1.19; albumin: 3.8 g/dL; direct bilirubin: 0.3 mg/dL; total bilirubin: 0.6 mg/dL; aspartate aminotransferase (AST): 28 IU/L; alanine aminotransferase (ALT): 5 IU/L; alkaline phosphatase (ALP): 163 U/L; blood urea nitrogen: 16 mg/dL; creatinine: 1.2 mg/dL; sodium: 131 meq/L; potassium: 4.1 meq/L; calcium: 10.5 mg/dL; C-reactive protein: 172.78 mg/L. The serum level of carcinoembryonic antigen (CEA) (< 5 ng/mL), alpha-fetoprotein (AFP) (< 15 ng/mL), and carbohydrate antigen 19-9 (CA 19-9) (< 37 U/mL) were 3.06 ng/mL, < 3 ng/mL, and 4.78, respectively. The plasma retention rate of indocyanine green (ICG) at 15 min was 0.21%. Subsequent abdominal ultrasonography (US) showed a mixed echoic mass about 7.8 cm occupying S5 of the right lobe of the liver (Figure 1A). The abdominal computed tomography (CT) showed a 9.5 cm × 7.0 cm irregular mass with inhomogeneous density and mild delayed enhancement in the central zone of the tumor near the gallbladder, at S5 of right lobe of the liver (Figure 1B). Angiography showed a large tumor with neovascularity and stretching the feeding arteries, located at S5 of right lobe of the liver. Diagnosis of a liver mass with hypervascularity at S5 of the right lobe of the liver was considered (Figure 1C).

In October 2005, we performed extended right lobectomy and cholecystectomy to remove the liver mass. Intra-operative finding showed non-cirrhotic liver with a 10 cm × 8 cm hard and white tumor mass with central necrosis located at S4, 5 and 6 (mainly S5) with no tumor rupture and no hemoperitoneum. The resection margin

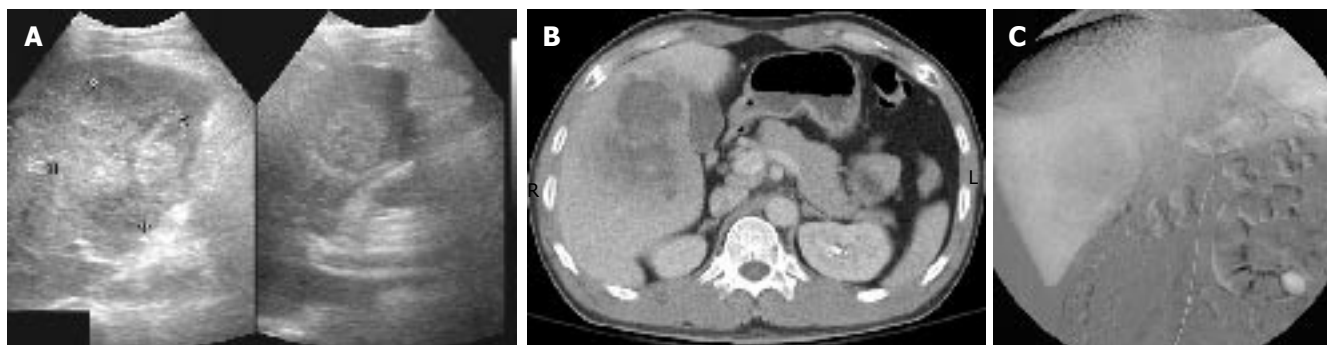


Figure 1 A: Abdominal ultrasonography (US) shows a mixed echoic mass about 7.8 cm occupying S5 of right lobe of the liver; B: Abdominal computed tomography (CT) shows a 7 cm x 9.5 cm irregular mass with nonhomogeneous density and mild delayed enhancement in the central zone of the tumor near the gallbladder, at S5 of right lobe of the liver; C: Angiography shows a large tumor with neovascularity and stretching the feeding arteries, located at S5 of right lob of the liver.

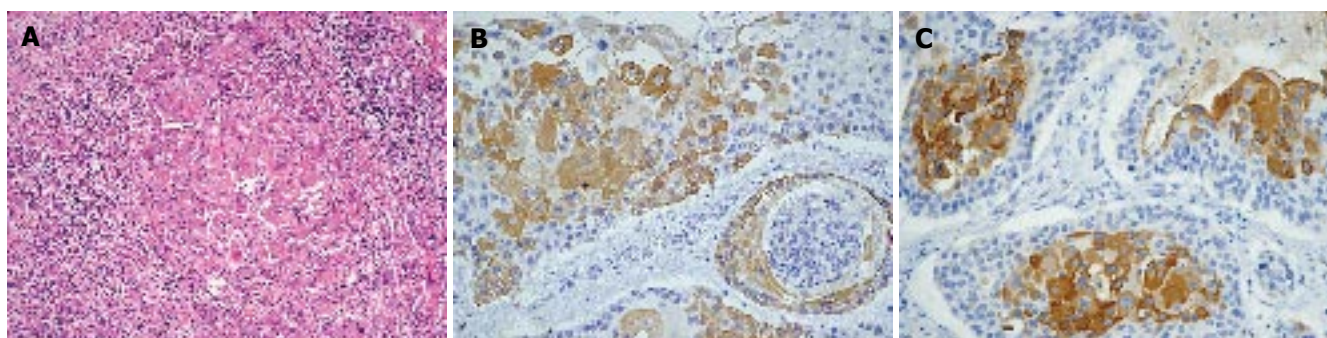


Figure 2 A: Microscopic findings of the resected liver tumor. The tumor is composed of squamous cells with keratinization (HE x 200); B: Squamous cells express strong positive CK14 staining immunohistochemically (IHC x 200); C: Squamous cells express strong positive CK10 staining immunohistochemically (IHC x 200).

was 2.0 cm in width.

Histopathological examination showed moderately differentiated SCC of the liver composed of squamous cells with keratinization (Figure 2A). An immunohistochemical study revealed positive for cytokeratin (CK) 10, CK14, CK19 and CEA. The occasional positivity for CK18 but negativity for thyroid transcription factor-I (TTF-1) indicated that it could be a primary SCC of the liver (Figure 2B, 2C). Subsequent panendoscopy showed negative findings for the esophagus. Chest CT revealed no mass over the lung field and ENT survey showed a negative finding in the oral cavity and nasopharynx. Postoperative course was uneventful and no tumor recurrence and distant metastasis developed during a 9-mo follow-up period.

DISCUSSION

Primary SCC of the liver is very rare. Although the carcinogenesis has been proposed of tumor transformation from chronic inflammation of the biliary epithelium or metaplastic and subsequent neoplastic transformation of pre-existing cysts of the liver^[1-3,8], as shown in this case, the true mechanism is still unknown.

Here, we reported the first case of SCC of the liver, presenting with a solitary solid tumor without parasitic infection and curatively treated by hepatic resection successfully. In this case, the pathology report

showed moderately differentiated SCC composed of squamous cells with keratinization. The positive staining of an acidic CK 14 indicated basal cells of keratinized squamous epithelium origin of the cancer cell. The strong, diffuse expression of biliary CK 19 confirmed the bile ductular ontogeny of the neoplastic cells. Thus tumor transformation from chronic inflammation of the biliary epithelium might explain its origin. Because TTF-I, an indicator of small cell carcinoma of the lung or thyroid is negative, metastatic lung or thyroid cancer to the liver could be ruled out. Clinically, panendoscopy, chest CT, and ENT examination revealed negative findings in the case. Taken together, the tumor could be a primary SCC of the liver^[10,11].

The prognosis of primary SCC of the liver is dismal with overall survival less than one year^[7-9], because the tumor is usually diagnosed late. Complete remission of poorly differentiated SCC after systemic chemotherapy (cisplatin and 5-fluorouracil) and surgery has been reported^[4]. Hepatic arterial injection of low dose anti-cancer drugs (cis-diaminedichloroplatinum) (CDDP) and 250 mg of 5-fluorouracil (5-FU) could achieve remarkably good response^[5]. As shown in the case, the postoperative course was smooth with disease free survival for 9 mo. Aggressive and meticulous follow up is needed. If tumor recurrence is detected, re-hepatectomy, systemic chemotherapy (cisplatin and 5-fluorouracil), or hepatic arterial infusion of low dose chemotherapy could

be considered^[4,5].

In summary, we describe the first case of a primary SCC of the liver presenting as a solitary solid tumor, and non-parasitic infection, successfully treated by surgical resection. Postoperative course was uneventful with 9-mo disease free survival. Aggressive and meticulous follow-up is warranted.

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CASE REPORT

Rare cause of ileus in the mesenteric cavity of terminal ileum: A report of three cases

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Abstract

Internal herniation is one of the rare reasons of intestine clog, which is hard to diagnose and usually needs an urgent surgical treatment. We report 3 patients with internal herniation in the mesenteric cavity of the terminal ileum. Besides intestinal congestion, they also had peritoneal irritation. Laparotomy revealed that herniation caused disorder in nutrition of the intestine and necrosis. The patients underwent subtotal small intestine resection and were discharged 10, 12 and 14 d after operation.

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Key words: Intestinal herniation; Ileus; Therapy

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INTRODUCTION

Internal herniation in intestine or in mesenteric cavities is a rare cause of intestinal congestions, accounting for less than 1% of intestinal congestions^[1]. Among the internal herniations, transmesenteric hernias constitute 12% of all intestinal hernias, 53% of intestinal hernias are seen in ileocecal area. In such hernias, there is no hernia bladder. Since the mesenteric cavities are narrow, strangulation is commonly seen and usually requires a surgical treatment^[1,2].

CASE REPORT

The first case was a 63-year-old male patient with the

complaint of common abdominal pain, intestinal gas and constipation, nausea and vomiting for 3 d. Physical examination revealed abdominal distension, defence, rebound tenderness and hypoactive bowel sounds. Other examinations were normal with no sign of previous abdominal surgery.

Laboratory examinations showed 11 000/mm³ leucocytes, 450 mg/dL glucose, 64 mg/dL BUN, 0.9 mg/dL creatine, other routine laboratory examinations were normal. Plain X-ray of the abdomen revealed a common air-fluid level on small intestine (Figure 1).

The second case was a 23-year-old male patient with the complaints of common abdominal pain, nausea, vomiting, intestinal gas and constipation. He had no history of any abdominal surgery. Physical examination showed abdominal sensitivity, defence and rebound tenderness, but no bowel sounds. Other examinations were normal. Laboratory examinations revealed 17 000/mm³ leucocytes, other laboratory examinations were normal. Plain X-ray of the abdomen showed a common air-fluid level in small intestine.

The third case was a 69-year-old male patient with the complaint of common abdominal pain, intestinal gas and constipation for a day. Physical examination showed abdominal distension and rebound tenderness, and a 10 cm × 10 cm mass in the lower right quadrant with hypoactive bowel sounds. Other examinations were normal. Laboratory examinations revealed 15 700/mm³ leucocytes, 82 mg/dL BUN, 0.7 mg/dL creatine. Plain X-ray of the abdomen, especially in the lower right quadrant displayed an intensified multiple air-fluid level in small intestine. All the three patients underwent surgical treatment with a pre-diagnosis of acute abdomen. Abdominal examination showed that most of the small intestines were passing through the cavity in the terminal ileum with a total disorder of nutrition and necrosis (Figures 2 and 3). All patients who underwent subtotal small intestine resection had diarrhea after surgery. They were treated with changed diet and medicine, and discharged 10, 12 and 14 d after operation.

DISCUSSION

The most common reason for intestinal congestion is the adhesions due to previous surgical operations (64%-79%)^[1]. The other reasons are incarcerated hernias in the abdominal wall, invagination, inflammatory bowel disease, trauma, bile stones, congenital atresia or stenosis, meckel diverticulum and internal herniations^[3,4].



Figure 1 Plain X-ray of the abdomen.

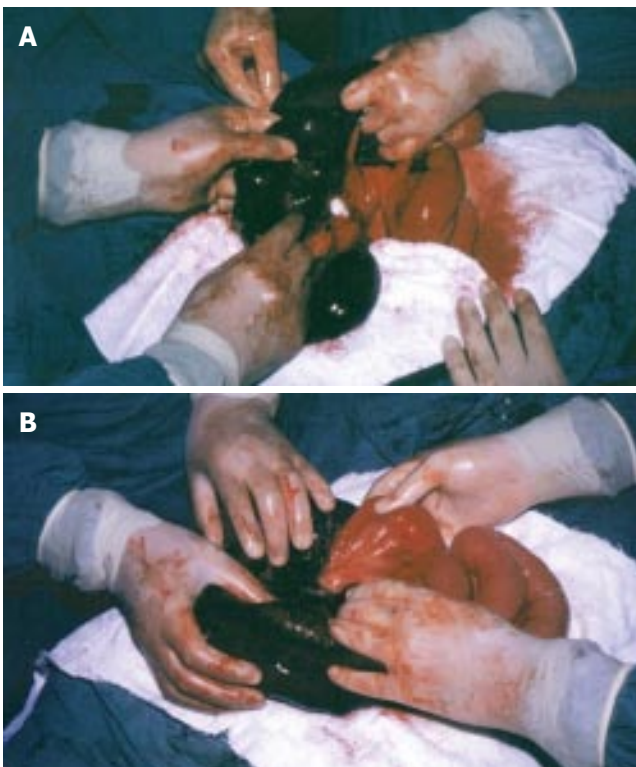


Figure 2 Disorder of nutrition(A) and necrosis (B) in terminal ileum.

Internal herniation, one of the rare causes, occurs as a result of herniation of the abdominal organs in openings or cavities of the peritoneum^[5]. Herniations are mostly due to anatomic structures such as paraduodenal fossa, and epiploic foramen, defects in mesentery and falciform ligaments^[3].

Also, intestinal rotation anomalies and peritoneal adhesions are causes of intestinal obstruction^[6]. The most common internal herniations according to their anatomic localizations are classified as follows: paraduodenal hernia, foraminal and pericecal, intersigmoidal, transmesenteric, transomental and retroanastomotic hernia^[5-8]. Mesenteric defect in the terminal ileum was found in our three patients who had no previous abdominal surgery or organ anomaly.

Internal herniations may cause chronic digestion disorders, postprandial pain and repetitive intestinal congestion.

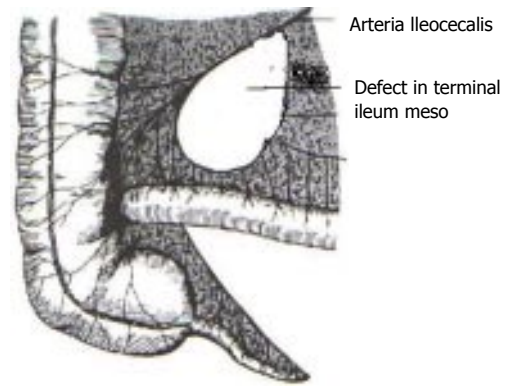


Figure 3 Defect in mesenteric cavity of terminal ileum (modified from figure published on Ankara Cerrahi Dergisi 2001; 3: 214).

Pain can be stopped by changing position. Vomiting is a common symptom and a quarter of the patients may have constipation. When the patients are given medical treatment due to these complaints, they sometimes can develop peptic ulcer or bile and biliary tract disease^[5]. Physical examination may show abdominal distension^[1]. The findings of laboratory and radiology support the diagnosis of intestinal congestion^[9]. Although no repetitive intestinal congestion was found in our patients, all of them had the main symptoms of intestinal congestion, such as abdominal pain, distension, intestinal gas, constipation, nausea and vomiting. In the preoperative period, the diagnosis is intestinal congestion, but it is almost impossible to diagnose internal herniation. Acute abdomen may develop into acute intestinal angina or malignancy in old patients, in which case laparotomy should be considered. Laparotomy can be performed in young patient with ileus and acute abdomen.

In conclusion, abdominal front wall herniation or malignancy should be considered first in adult patients with intestinal obstruction and previous surgical operations. Intestinal congestion may be caused by internal herniation in patients without such symptoms.

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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
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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
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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]

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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]

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- 9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Chapter in a book (list all authors)

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Human hepatic sinusoidal endothelial cells can be distinguished by expression of phenotypic markers related to their specialised functions *in vivo*

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Abstract

The hepatic sinusoids are lined by a unique population of hepatic sinusoidal endothelial cells (HSEC), which is one of the first hepatic cell populations to come into contact with blood components. However, HSEC are not simply barrier cells that restrict the access of blood-borne compounds to the parenchyma. They are functionally specialised endothelial cells that have complex roles, including not only receptor-mediated clearance of endotoxin, bacteria and other compounds, but also the regulation of inflammation, leukocyte recruitment and host immune responses to pathogens. Thus understanding the differentiation and function of HSEC is critical for the elucidation of liver biology and pathophysiology. This article reviews methods for isolating and studying human hepatic endothelial cell populations using *in vitro* models. We also discuss the expression and functions of phenotypic markers, such as the presence of fenestrations and expression of VAP-1, Stabilin-1, L-SIGN, which can be used to identify sinusoidal endothelium and to permit discrimination from vascular and lymphatic endothelial cells.

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INTRODUCTION

The liver has a unique dual blood supply receiving blood from both the portal vein and the hepatic artery. Unlike other organs, which are supplied by arterial blood through arterioles, the liver receives venous blood at low pressures through the portal vein as well as arterial blood *via* the hepatic artery. The intrahepatic portal venous system consists of conducting and distributing systems that ensure blood is carried throughout the parenchyma and evenly delivered to individual hepatocytes *via* the sinusoidal network^[1]. A uniform and strict branching pattern appears at the level of the terminal vessels and is maintained throughout the distributing system. The first order branches of the distributing system arise from the terminal branches of the conducting system and give rise to second order vessels of approximately 70 μ m diameter that correspond to the terminal portal vein branches seen in portal tracts. Third order vessels arise from these and correspond to the septal or interlobular branches. These exhibit a classical sinusoidal appearance, lack a connective tissue sheath and have no basement membrane. The distributing system then branches into the hexagonal lobule before draining into the hepatic vein. The hepatic artery supplies 25% of hepatic blood flow and provides blood to both the parenchyma and the portal tracts. Axial hepatic arterial branches run parallel to the conducting portal veins and terminate in inlet venules, terminal portal veins and the sinusoids, thereby supplying blood to the parenchyma. The axial arteries also give rise to peribiliary branches that supply the accompanying bile ducts and portal interstitium. These arteries form the peribiliary plexus consisting of efferent and afferent capillaries that wrap around the surface of bile ducts. Small channels from the peribiliary plexus drain into the sinusoids or portal vein branches. Thus, complex anastomoses exist between the axial arteries and peribiliary arteries^[2].

ISOLATION AND CULTURE OF HEPATIC SINUSOIDAL ENDOTHELIAL CELLS

Isolated cultures of hepatic sinusoidal endothelial cells present a valuable tool for the study of liver physiology and pathophysiology. Most published studies used cells isolated from rodent livers and have defined the role of fenestrations and specific receptors in uptake and

processing of circulating factors including pathogens (reviewed in^[3]). Most investigators use a combination of enzymatic digestion and density gradient centrifugation to isolate HSEC from liver tissue although there is considerable variation in the protocols used by different groups and for cells from different species. HSEC from rodents are commonly isolated by enzymatic digestion, either by perfusion of an intact organ with an enzymatic cocktail or by mechanical disruption followed by enzymatic digestion^[4-7]. The cell suspension generated by such methods is fractionated using differential centrifugation techniques, including counterflow elutriation or density gradient centrifugation. Endothelial cells are then grown on matrix coated flasks in selective growth media containing growth factors.

Similar methods can be used to isolate HSEC from human liver samples^[8-10] and between 10^3 - 10^6 cells have been isolated from whole livers unsuitable for transplantation^[9]. However, the limited access to intact human liver means that most groups use diseased tissue removed at transplantation or surgical resection or biopsy specimens that generate low numbers of cells per isolation. Nevertheless, successful isolation is aided by the large numbers of HSEC relative to other vascular endothelial cells present in the liver. Some researchers further reduce the potential of contaminating vascular endothelium by excising visible large vascular structures from the liver tissue prior to enzymatic digestion. Although several different isolation procedures have been described these are not all equally effective in our hands. Our experience, and that of other groups, suggests that counterflow elutriation alone is not useful for selection of human HSEC from a mixed non-parenchymal cell preparation as most of the cells have similar centrifugal densities^[6,11]. This led us to routinely include a step of immunomagnetic depletion to remove common contaminating cell populations, such as biliary epithelial cells, followed by positive selection of endothelial cells using antibody against CD31 (see later)^[10]. Similar methods using magnetic beads coated with *Evonymus europaeus* agglutinin have been used to isolate HSEC from the liver of other primates^[12] and there are now commercial antibody-based magnetic kits for isolation of rodent HSEC. Despite these refinements there is considerable variability in the yield and viability of cells obtained from diseased human liver tissue. Thus, although there are compelling reasons to study human HSEC *in vitro* low cell yields mean that it is difficult to use primary cells without passage.

PROBLEMS WITH EXISTING PHENOTYPIC MARKERS AND USE OF HSEC IN MONO-CULTURE

As scientific technology advances it is possible to carry out increasingly complex genetic, proteomic and functional analyses on cells grown in culture. However such techniques require highly purified populations of cells of defined phenotype. Some of the methods previously used to confirm HSEC purity and phenotype, such as AcLDL uptake^[8] and binding of *Ulex* lectin^[8], are

not specific. For example, other hepatic cell populations, including dendritic cells, take up AcLDL and *Ulex* lectin binds to fucosylated receptors on both Kupffer cells and HSEC^[13]. Similarly, endothelial cells share many cell surface receptors with leukocytes, including CD31, CD4^[14], CD11b, and CD11c^[11], which may contaminate endothelial cell preparations in culture. Care also needs to be taken when using antibody staining to define HSEC phenotype because HSEC express high levels of FcγR^[15] allowing them to bind antibodies non-specifically. In light of such problems, it has been suggested that the presence of open fenestrations arranged in sieve plates is the only true marker of hepatic sinusoidal endothelial cells^[16]. These pores are indeed classic features of liver sinusoidal endothelial cells *in vivo* but present problems when used to identify cells *in vitro* (see below).

All of these problems are compounded by the fact that HSEC are most commonly cultured as a monolayer of cells on matrix-coated tissue culture plates *in vitro*. This perturbs the normal morphology of the cells and they become flattened and rapidly lose fenestrations. Part of this effect may be the loss of paracrine signals from other cells of the sinusoid that maintain the phenotype and differentiation of HSEC *in vivo*. For example, crosstalk between hepatic sinusoidal endothelial cells and closely juxtaposed hepatocytes is essential for the maintenance of sinusoidal endothelial cell growth and differentiation. This is demonstrated by studies where implantation of foetal liver fragments into quail chorioallantoic membrane resulted in the acquisition of a sinusoidal phenotype by the chorioallantoic microvessels^[17] and also *in vitro* studies where co-culture of HSEC with other liver cells resulted in a more stable endothelial phenotype and function^[18]. Thus, markers used to determine phenotypes of HSEC must take into account alterations in phenotype as a consequence of culturing cells in isolation in the absence of local paracrine signals.

FENESTRATIONS

Endothelial cells throughout the adult organism are derived from common early embryological precursors and have broadly similar functions and histological appearance. However, there is important organ and tissue-specific heterogeneity that results in phenotypic and functional variations (reviewed in^[19]). For example, high endothelial venules in lymph nodes are lined by morphologically and phenotypically distinct endothelial cells that have the unique ability to promote the recruitment of naïve lymphocytes whereas lymphatic endothelium express several receptors that allow uptake of macromolecules found in lymph^[20]. Sinusoidal endothelial cells are found in the spleen and bone marrow, as well as in the liver, and in all these sites they have a minimal basement membrane and lack classical tight junctions. Hepatic sinusoidal endothelium differs from sinusoidal endothelium in these other beds by its discontinuous nature, being interspersed with kupffer cells and by the presence of open fenestrations arranged in sieve plates^[16].

The vascular architecture in the human liver develops by 17-25 wk of gestation, and the sinusoids acquire their

Table 1 The expression of classical markers of endothelial phenotype by human sinusoidal endothelial cells

Marker	Extra-hepatic endothelial expression	Sinusoidal endothelial cells <i>in vivo</i>	Sinusoidal endothelial cells <i>in vitro</i>	Problems with use in phenotyping?
CD31	Vascular and lymphatic endothelial cells	Yes, but at low levels	Yes	Widely expressed on all EC thus not specific for HSEC
vWF	Vascular endothelial cells	Controversial, Yes	Yes	Widely expressed on all EC thus not specific for HSEC
Ulex lectin binding	Endothelial cells	Yes	Yes	Widely expressed on all EC thus not specific for HSEC
Uptake of AcLDL	Endothelial cells	Yes	Yes	Also taken up by macrophages in liver and other EC
CD34	Vascular and lymphatic endothelial cells	No	Absent or low	May be upregulated during capillarisation or with passage <i>in vitro</i>
E-Selectin	Vascular endothelial cells	Low or absent under normal conditions	Low, can be upregulated by cytokines	Widely expressed on activated vascular EC, not specific for HSEC
Pal-E antigen	Vascular endothelial cells	No	No?	May be upregulated during capillarisation
CD105/endoglin	May be upregulated during capillarisation	Yes	Yes	Widely expressed on all EC, also by stellate cells and fibroblasts in liver

While many of the above markers are indeed expressed on hepatic sinusoidal endothelial cells and provide a means of confirming “sinusoidal endothelial identity”, none is specific to sinusoidal endothelial cells.

distinctive fenestrated phenotype by wk 20 (reviewed in^[21]). The fenestrations act as a ‘dynamic filter’^[3] allowing macromolecules in blood controlled access to parenchymal cells^[22]. Evidence from animal studies suggests that fenestrations constitute up to 40% of the cell and that the size, distribution and clustering of the pores in sieve plates varies with the zonal distribution of the endothelium^[23] and across the endothelial surface. Although normal hepatic sinusoidal cells in most mammals are characterised by the presence of fenestrations (reviewed in^[3]), caution must be exercised when translating these observations to human cells. Studies of fenestrations in human liver samples are rare, and suggest that the number and size of fenestrations differs from that observed in other mammals^[3]. Furthermore, the number of fenestrations per endothelial cell decreases in disease^[24,25], following viral infection^[22] or with ageing^[26]. During cirrhosis and chronic hepatitis, HSEC develop a more vascular morphology and produce a basement membrane in a process known as ‘capillarisation’ (reviewed in^[27]). This is associated with increased expression of CD31 and VCAM-1 and loss of fenestrations^[27]. These changes may impede the transfer of materials to and from the parenchyma and contribute towards regional hepatocyte hypoxia. Fenestrations are not unique to hepatic EC but are found in endothelium in endocrine glands, kidney, gastrointestinal tract, choroid plexus, lymphatic organs such as the spleen and are sometimes observed in tumour vasculature. Many studies have implicated VEGF as an essential factor for regulation of fenestrations in these organs (reviewed in^[28]).

Thus considerable variations in the number, size and localisation of fenestrations are seen among species and also in health and disease. The situation becomes more complex when cells are removed from the hepatic microenvironment and cultured *in vitro*. The fenestrations documented in freshly isolated rat HSEC begin to disappear within 48 h of cell culture^[29] and are almost gone within a week^[30]. We have made similar observations with human cells from normal livers and also find very few

fenestrated cells when HSEC are isolated from cirrhotic livers (Lalor and Adams unpublished observations). However, the number of fenestrations on rat HSEC can be maintained *in vitro* by the addition of VEGF and by culturing cells on extracellular matrix constituents, such as collagen, that are secreted by endothelial cells^[29,31]. Both human and rodent HSEC need growth factors and attachment to appropriate extracellular matrix molecules to survive and will rapidly undergo apoptosis in the absence of these. Thus in order to maintain cell survival, cultured HSEC must be grown in the presence of VEGF which induces and maintains expression of fenestrations as well as promoting HSEC proliferation. VEGF is also a growth factor for vascular endothelial cells which induces the production of matrix molecules essential for survival and proliferation^[32]. At higher concentrations, VEGF can induce the formation of fenestrations in vascular endothelial cells^[28,32,33] and, although these pores are not organised into sieve plates, they can be very difficult to distinguish from the fenestrations that characterise HSEC. This inducibility of fenestrations in vascular endothelial cells together with the impracticality of using electron microscopy for routine phenotyping means that the presence of fenestrations alone cannot be used to define HSEC in most experimental situations.

NORMAL ‘ENDOTHELIAL’ CELL PHENOTYPE AND FUNCTIONS

HSEC form a single cell barrier between the hepatocytes and the bloodstream and are strategically situated to interact with leukocytes and other blood constituents. The cells produce a minimal basement membrane^[34], which is mostly composed of type IV collagen in normal liver^[35], and have a high endocytotic capacity^[3]. They express some markers that are common to all endothelial cells and these provide a useful means to positively identify a cell of ‘endothelial’ lineage (Table 1).

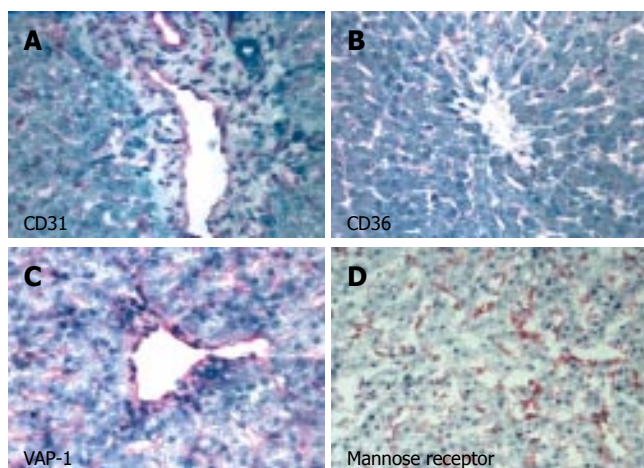


Figure 1 Human hepatic sinusoidal endothelial cells *in situ* express the classical endothelial phenotypic markers CD31 (A) and CD36 (B) as well as more recently identified markers such as VAP-1 (C) and Mannose receptor (D). Images represent immunohistochemical staining of human liver sections using specific primary antibodies in an indirect immunoperoxidase protocol with haematoxylin counterstain. Positive staining of sinusoidal endothelial cells is indicated by pink pigmentation. CD31, CD36 and VAP-1 are observed on both portal vessel endothelial cells and sinusoidal endothelium, whilst the mannose receptor is localised on sinusoidal endothelial cells and Kupffer cells.

CD31

CD31 or PECAM-1 is an abundantly expressed membrane glycoprotein member of the immunoglobulin superfamily (reviewed in^[36]). It is constitutively expressed on endothelial cells and some haematopoietic cells and has functions in cell adhesion and signalling. Expression of CD31 is used widely as a marker of 'continuous' or classical vascular endothelium, but expression of CD31 by hepatic sinusoidal endothelial cells remains controversial. In rodent studies, CD31 has been localised to normal sinusoidal endothelial cells using a variety of techniques^[4,37] and has been shown to be downregulated following CCl₄ or TNF α mediated liver injury^[37]. However, other studies suggest that the protein is absent under normal conditions^[34] and indeed a lack of CD31 has been used to characterise rodent HSEC^[7].

The situation is similarly complex in human HSEC. During human embryonic development CD31 is absent from HSEC until wk 25. In the adult, CD31 has been reported as present on HSEC by FACS and immunohistochemistry on sections from cirrhotic liver but is minimally present on normal liver^[8] or alternately present on normal liver and enhanced on cirrhotic liver^[38]. Our own data show that CD31 is expressed by both normal and diseased liver HSEC (Figures 1 and 2). In general, it seems that HSEC express lower levels of CD31 than vascular EC^[16] and our observations). However, one needs to interpret immunohistochemical analysis with caution because CD31 is present on kupffer cells in sinusoids. Studies on isolated HSEC using both PCR and antibody-based assessment of characteristic endothelial markers, such as CD31 and vWF (see below) in parallel, show varying results^[39]. Some studies suggest that subcellular localisation of CD31 can be used as a marker of HSEC phenotype to indicate whether cells have dedifferentiated in culture^[16].

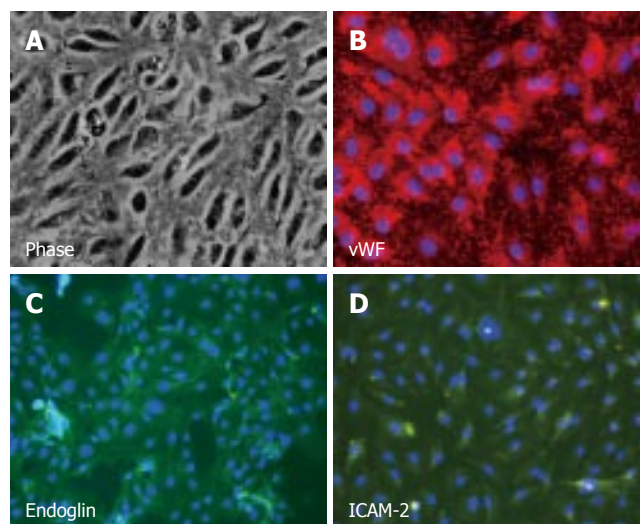


Figure 2 Isolated, cultured human hepatic sinusoidal endothelial cells exhibit classical morphology under phase contrast microscopy (A), and stain positively with antibody directed against endothelial phenotypic markers. Images represent immunofluorescent staining of cultured cells using specific primary antibodies in an indirect fluorescent protocol with DAPI nuclear counterstain (blue). Positive staining for vWF (B) is visualised using a Texas Red-labelled secondary antibody, whilst expression of endoglin (CD105, C) and ICAM-2 (D) are visualised with a FITC-conjugated secondary antibody (green).

Thus cells with cytoplasmic CD31 are 'normal' whereas dedifferentiated/capillarised EC demonstrate increased membranous expression.

Von Willebrand Factor (vWF)

vWF is a multimeric glycoprotein that binds and stabilises the coagulation factor FVIII as well as supports the adhesion of platelets to subendothelial structures during vascular damage. It is expressed by both platelets and endothelial cells and is often used as a marker to identify endothelium. In most vascular endothelial cells, von Willebrand Factor is stored in cytoplasmic vesicles called Weibel Palade bodies. Expression of vWF varies between different vascular beds *in vivo*^[40] and particularly low levels are observed in the liver, most of which is detected in vascular rather than sinusoidal endothelial cells. The low levels of vWF detected in HSEC are consistent with the reported lack of Weibel Palade bodies. However, definitive evidence supporting the presence or absence of these structures is also lacking. Studies in mice suggest that HSEC contain Weibel-Palade bodies^[41] and produce vWF at the mRNA and protein level^[4,11,41]. Other groups working with rat HSEC report that vWF is not expressed in normal rat cells^[42] and these findings are supported by porcine and rat studies showing absence of Weibel-Palade bodies in HSEC^[9,43]. In human cells, vWF expression has been reported in both normal^[8,44] and diseased samples^[45] and we and others^[8] have demonstrated expression on passaged, cultured HSEC *in vitro* (Figure 2).

E-Selectin

E-Selectin is a member of the selectin family of adhesion molecules that supports leukocyte binding. Expression of E-Selectin is restricted to cells of endothelial lineage^[46]

and is induced by inflammation *in vivo* and exposure of endothelial cells to proinflammatory cytokines and LPS *in vitro*. The ability to express E-selectin can thus be used to define endothelial cells in culture. However, expression of E-selectin is restricted to vascular endothelial cells in the normal liver^[47], although it may be upregulated on sinusoidal endothelium in disease and during metastatic processes^[48] and animal studies demonstrate a minimal role for E-selectin in leukocyte recruitment to liver tissue^[49]. However, we have reported expression of functional E-Selectin on cultured cytokine-stimulated human HSEC suggesting that HSEC can express E-Selectin under restricted circumstances *in vivo* and *in vitro*^[18]. Recent studies demonstrating that E-Selectin expression by HUVEC in response to TNF α is reduced by pretreatment with HGF suggest that paracrine factors from adjacent hepatocytes may suppress E-selectin in the sinusoids *in vivo*^[50].

Binding of lectins and Acetylated LDL uptake

The ability to bind Ulex lectin and take up acetylated LDL is often used to define HSEC. Ulex lectin, from the gorse family of plants, binds α -L fucose containing receptors and is commonly used as a histological marker for endothelial cells, although in some tissues it also binds epithelial structures^[51]. In the liver, different lectins bind differentially within the vasculature. Concanavalin A binds with equal affinity for all segments of the microvasculature whereas wheat germ agglutinins show preferential binding to the sinusoidal vasculature as a consequence of differences in distribution of glycosylated ligands throughout the acinus. In most studies Ulex lectins do not bind preferentially to sinusoidal endothelium as a consequence of the relative paucity of α -L fucose motifs. The ability of wheat germ lectins to bind sinusoidal endothelium has led to their use in selectively purifying sinusoidal EC and their preference for periportal HSEC has even led to the suggestion that they can be used to differentially purify periportal versus perivenous HSEC^[5]. Staining with Ulex lectin is increased in disease and is particularly pronounced during capillarisation of the sinusoids^[52]. Thus although binding of Ulex lectin is indicative of an endothelial phenotype, it is not restricted to 'sinusoidal' endothelial cells^[52] and is not a good marker of HSEC.

The liver is the major site for the scavenger receptor-mediated clearance of lipoproteins from the circulation. Acetylated LDL is mainly cleared by hepatic endothelial cells^[53] by binding to scavenger receptors including scavenger receptor class A (SR-AI/II). However, these receptors are expressed by both HSEC and macrophages^[54] and the ability to take up acetylated LDL is common to many extrahepatic endothelial cell populations, again reducing the specificity of acLDL uptake as a characteristic property of HSEC.

CD34

CD34 is a type 1 transmembrane sialomucin expressed by haematopoietic stem cells, capillary and lymphatic endothelial cells. CD34 is absent from most sinusoidal endothelial cells in normal liver but expression

increases^[8,27,55] during capillarisation in chronic inflammatory disease and in the sinusoidal-type vasculature within hepatocellular carcinomas^[56,57]. CD34 expression has also been shown to increase in other tissues including the rheumatoid joint and at sites of neolymphoid development during chronic inflammation. We have described CD34 positive lymphatic-like vessels in portal associated lymphatic tissue in chronic inflammatory liver diseases including PSC^[58] and hepatitis C (Heydtmann 2006 *J Immunol* in press). However, CD34 is not expressed on most HSEC in non-inflamed tissue *in vivo* and is absent from low passage human HSEC *in vitro*.

Pal-E Antigen

The antigen recognised by the Pal-E antibody is a widely used marker of vascular endothelial cells^[59-61]. The identity of the protein has been proposed as either a secreted form of vimentin produced by endothelial cells^[61] or a protein designated PV-1/FELS (plasmalemmal vesicle-1/fenestrated endothelial-linked structural protein)^[60]. PV-1 is particularly interesting in the context of HSEC. As its name implies, PV-1/FELS is expressed by fenestrated endothelial cells in the kidney and pancreas^[62] but it is absent from fenestrated hepatic sinusoidal endothelial cells^[60,62] and is restricted to vascular endothelium and neovessels in areas of capillarisation in chronic liver disease and hepatocellular carcinoma^[52,58].

Vascular Endothelial-Cadherin (VE-Cadherin)

Cadherins are a family of adhesion molecules that demonstrate cation- dependent homophilic and heterophilic binding. Endothelial cells express at least three cadherins: N-, P-, and VE-cadherin. VE-cadherin is localised to the inter-endothelial cell junction where it is an essential part of the adherens junctions that maintains endothelial permeability, monolayer integrity, morphogenesis and angiogenic responses. Most studies suggest that sinusoidal endothelium in normal liver lacks VE-cadherin or expresses it at low levels, although it can be detected in chronic inflammation^[38,63]. It seems likely that the relative lack of VE-cadherin on HSEC is a consequence of the absence of classical adherens junctions between HSEC and this is consistent with a lack of other junctional proteins, including vascular endothelial junctional adhesion molecule (VE-JAM/JAM-2), which is a member of the immunoglobulin superfamily structurally similar to JAM-1, which is absent from foetal and adult liver^[64].

CD105/endoglin

CD105 (endoglin) is a hypoxia-inducible protein that is widely expressed on endothelial cells and is upregulated during angiogenesis. It is a receptor for transforming growth factor (TGF) β 1 and β 3 and modulates TGF- β signalling by interacting with TGF- β receptors I and/or II (for review see^[65]). CD105 has been used as a marker of angiogenesis, particularly in tumour tissue^[66], and because it is a transmembrane molecule, it has been used in antibody-mediated positive selection strategies for endothelial cell isolation. However, expression of the

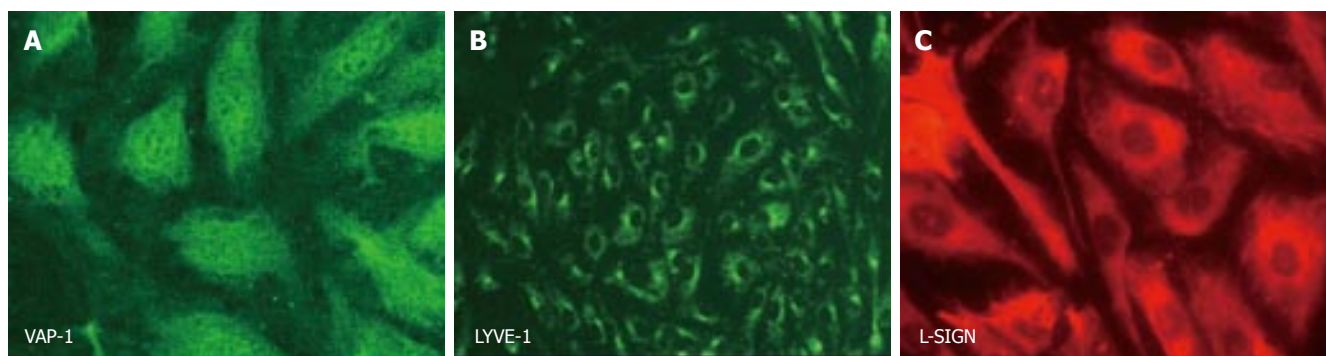


Figure 3 Cultured human hepatic sinusoidal endothelial cells stain positively with antibody directed against 'non-classical endothelial' phenotypic markers. Images represent immunofluorescent staining of cultured cells using specific primary antibodies in an indirect fluorescent protocol. Expression of VAP-1 (A) and LYVE-1 (B) are visualised with a FITC-conjugated secondary antibody (green), whilst positive staining for L-SIGN (C) is visualised using a Texas Red-labelled secondary antibody.

molecule is not restricted to endothelial cells^[65], and in the liver, expression has been reported in both stellate cells and myofibroblasts^[67]. We have demonstrated that CD105 is expressed on hepatic sinusoidal endothelial cells (Figure 2) but again emphasise that this is not a tissue- or cell lineage-specific marker and urge caution when using it as a phenotypic identifier.

NEWER MARKERS OF ENDOTHELIAL PHENOTYPE RELATE TO THE FUNCTIONS OF HSEC WITHIN THE LIVER MICRO-ENVIRONMENT

As well as exhibiting features characteristic of all endothelial cells, hepatic sinusoidal endothelial cells fulfil many specific features within the liver environment. These include providing a barrier to minimise access of blood-borne material into the parenchyma and specific protein/antigen uptake and presentation. The embryonic origins of these cells and their similarity with lymphatic endothelial cells (see later) mean that they express several markers that are not present on vascular endothelium and these can be used to distinguish them from other endothelial cells. Thus, it is possible to use protein markers related to these specific origins and functions to confirm the phenotype of HSEC *in vitro*.

Scavenger functions/lipid uptake functions of EC

The exposure of sinusoidal endothelial cells to blood originating from both the systemic circulation and the gut means that HSEC are strategically situated to remove and recycle blood-borne proteins and lipids. In combination with Kupffer cells, HSEC constitute the most powerful scavenger system in the body^[68]. The uptake of solutes is facilitated by the presence of fenestrae, the lack of a classical basement membrane and the expression of multiple scavenger receptors that allow them to bind and take up specific classes of molecules. These properties facilitate bidirectional transfer of materials to the parenchyma. Many of the scavenger receptor proteins can be used to determine the phenotype of HSEC.

The link family of proteins has recently been described

as scavenger receptors responsible for clearance of a variety of proteins, including advanced glycation end products, modified LDL and bacteria^[69]. Two members of this protein family, Stabilin-1 and -2, are constitutively expressed by hepatic sinusoidal endothelial cells^[69,70]. Stabilin-2 is the major lymph node and liver hyaluronan and glycosaminoglycan scavenger receptor whilst Stabilin-1 (also called Feel-1 or CLEVER-1^[71-73]) is a more promiscuous scavenger receptor. In common with many other scavenger-type receptors, these proteins are present on sinusoidal endothelial cells in spleen and lymph node as well as the liver. Most of the scavenger functions assigned to this molecule relate to endocytosis of hyaluronic acid, acetylated LDL and glycation end products, but there is also evidence to support roles for Stabilin-1 in leukocyte adhesion and tumour metastasis^[69,74]. Another member of the link protein family is LYVE-1, an endothelial hyaluronan receptor predominantly restricted to lymphatic endothelial cells (reviewed in^[75]). Putative functions for LYVE-1 include the uptake of hyaluronic acid and regulation of leukocyte adhesion or migration within the lymphatic circulation. Interestingly, hepatic sinusoidal endothelial cells also express LYVE-1 constitutively^[76] (Figure 3) with evidence of a zonal distribution, the highest levels being detected in acinar zone 2. This hyaluronan receptor is present on both normal and diseased human HSEC, although lower levels are observed in cirrhosis and the protein is absent in HCC^[76]. Expression is also seen on portal-associated lymphatics in chronic liver disease^[58,76].

The liver is the major site for synthesis and metabolism of cholesterol and scavenger receptors of class A (SR-A) on both KC and HSEC^[77] are responsible for the uptake of oxidised/acetylated LDL, which is subsequently passed on to hepatocytes. Another HDL/LDL receptor, CD36^[78], also known as GPIV, is expressed at high levels on platelets, monocyte/macrophages and vascular endothelial cells. In the liver, CD36 is strongly expressed on sinusoidal endothelial cells^[79] where it fulfills multiple functions including acting as a scavenger receptor for oxidised lipid^[80] and as an adhesion receptor for red blood cells infected with malarial parasite^[81]. There are two alternatively spliced members of the scavenger receptor B family (SR-BI and -BII). Scavenger receptor-B1 is expressed by HSEC and

is responsible for the uptake of HDL cholesterol esters to liver parenchymal cells and also acts as a coreceptor for HCV infection^[82].

The calcium-dependent C-type lectins, Dendritic Cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) or CD209 and the related molecule Liver/lymph node-specific intercellular adhesion molecule-3-grabbing nonintegrin (L-SIGN) or CD209L (Figure 3), are constitutively expressed on hepatic endothelial cells^[83]. DC-SIGN is expressed at high levels on myeloid dendritic cells in tissues where it interacts with ICAM-3 on T cells as part of the immunological synapse as well as being an attachment factor for HCV, ebola virus, CMV, HIV and other lentiviruses (reviewed in^[84,85]). We have recently reported that DC-SIGN is present on hepatic sinusoidal endothelial cells and that expression is increased in response to treatment with cytokines including IL-4^[86]. DC-SIGN on endothelial cells acts as an attachment factor for HCV but does not mediate HCV entry directly but rather enhances infection of hepatocytes in trans. DC-SIGN is absent from most other vascular beds, although it has been reported on a brain microvascular cell line^[87].

Liver/lymph node-specific ICAM-3-grabbing integrin (L-SIGN), otherwise known as DC-SIGN-related (DC SIGNR) (CD209L), shares 77% amino acid homology with DC-SIGN. Like DC-SIGN it can also bind ICAM-3, HIV and HCV. L-SIGN is strongly and constitutively expressed on sinusoidal endothelial cells in the liver and on endothelium in lymph nodes but not on DCs or on endothelium in other tissues. L-SIGN is thus an excellent marker of liver endothelium^[44,88]. A related molecule, Liver and Lymph node Sinusoidal Endothelial C-type lectin (LSEctin) is also expressed on sinusoidal endothelial cells and has recently been demonstrated to mediate attachment of filovirus and coronavirus particles^[89]. Thus sinusoidal endothelial cells^[90] can bind a wide variety of pathogens after which they pass hepatotropic viruses on to adherent hepatocytes in trans^[91] thereby concentrating viral pathogens within the liver.

Antigen presentation by HSEC

As well as being equipped with scavenger receptors that facilitate efficient uptake of viruses and potential antigens, HSEC also have the ability to phagocytose particles and to present antigen to lymphocytes (reviewed in^[92-94]). There is evidence that such interactions are important for generating immunological tolerance to gut-derived antigens although recent work suggests that local antigen presentation cannot explain liver tolerance and that, on the contrary, the liver may be an excellent priming site for naive CD8⁺ T cells^[95]. Antigen presentation is facilitated by the expression of MHC class I and II^[96,97] molecules together with co-stimulatory molecules such as CD40 and more contentiously CD80 and CD86^[98]. These receptors are upregulated on HSEC in fulminant liver failure^[99] and may contribute to disease pathogenesis by allowing ongoing presentation of stimulatory antigen. HSEC also express the mannose receptor (Figure 1), a 175 kDa transmembrane glycosylated protein involved in uptake of Ag by both DCs and HSEC^[97]. Competitive inhibition

of this receptor by mannan reduces antigen-specific T cell activation by murine HSEC^[97].

Similarities between lymphatic endothelial cells and sinusoidal endothelium

Both the liver and pancreas develop from buds of the embryonic endoderm^[100], however, the vasculature components of the liver have distinct origins. The portal vessels are derived from vitelline veins whereas the sinusoids develop from the capillary vessels of the septum transversum and acquire their distinctive fenestrated phenotype by wk 20 of gestation (reviewed in^[21]). From this point onward, sinusoidal endothelial cells remain functionally and phenotypically distinct from the other vascular endothelial cells in the liver and express several receptors that are otherwise confined to lymphatic endothelial cells that are derived from buds from the cardinal vein. Hence, both lymphatic and sinusoidal endothelial cells have minimal basement membranes, loosely organised cell junctions and constitutively express LYVE-1 and VAP-1 (SSAO/AOC3) but lack CD34 (reviewed in^[101]). VAP-1 is a type II transmembrane protein that can support leukocyte adhesion *via* interactions with sialic acid rich side chains. It is also an amine oxidase and enzyme activity is also involved in regulating leukocyte adhesion and transmigration^[101]. VAP-1 is expressed on all vascular compartments within the liver (Figures 1 and 2) where it supports the adhesion and transmigration of leukocytes^[10,102]. The only extrahepatic site where VAP-1 is constitutively expressed at high levels is endothelial cells in high endothelial venules within lymph nodes^[103] where again it is proposed to have a role directing the adhesion of lymphocyte populations^[104]. Similarly both lymphatic endothelium and HSEC express the Reeler gene product Reelin^[105]. Reelin is a secreted glycoprotein with roles in embryonic development and organisation. Expression is restricted during embryogenesis but in the adult organism high levels are detected on lymphatic endothelial cells and within the sinusoids localised either to stellate cells^[106] or HSEC^[105]. This has led to the hypothesis that reelin may be involved in the regulation of lymphoangiogenesis or regulation of lymphatic endothelial phenotype and thus may have similar roles within the liver sinusoids.

Thus there are many similarities between HSEC and lymphatic endothelial cells and some antigens originally defined on lymphatic endothelium can also be used to differentiate between HSEC and vascular endothelial cells in the liver. It is possible to exclude contamination with lymphatic EC in HSEC cultures on the basis that LYVE-1 positive HSEC do not express PROX -1, a transcription factor found exclusively in lymphatic EC^[107].

CONCLUSIONS

Cultures of endothelial cells are valuable tools to investigate mechanisms of liver physiology and pathophysiology *in vitro*. However, the study of endothelial cells *in vitro* is complicated by the marked heterogeneity of endothelial cells between and within different organs and the tendency for cells to lose tissue-specific markers

when cultured *in vitro*. Although all endothelial cells share some characteristic features (as described in the first part of this review), there is a need for specific markers or combinations of markers that define distinct populations of endothelial cells. To date, the study of HSEC *in vitro* has been hampered by the lack of specific markers that can conclusively identify these cells and discriminate them from vascular or lymphatic endothelial cells. To some extent this remains the case, since many classical endothelial markers are widely expressed (Table 1) and there is considerable reported variability in detection of phenotypic markers between animal and human systems (eg CD31 and vWF). To date there is no known single molecule that is only expressed on hepatic sinusoidal and no other type of endothelia. However, the increasing knowledge of endothelial receptors is providing us with a larger and better defined set of phenotypic makers. In addition to their use in phenotyping or sorting/ selecting specific endothelial cells for culture, receptors that show tissue-specific expression provide clues to specific functions of the cells being studied. Examples of this are the large number of scavenger receptors expressed by HSEC. Cultured HSEC do exhibit some useful identifying features, however. Very low passage cells retain fenestrations *in vitro* for a short time but these rapidly disappear within a passage or two in culture^[8,16], as does expression of VAP-1^[10]. Apart from these changes, however, the cells remain relatively phenotypically stable for 7-8 passages^[8] and can be identified by expression of CD31, LYVE-1, DC-SIGNR(L-SIGN), Stabilin-1 and lack of CD34 and PROX-1^[107]. These markers confirm endothelial identity, while excluding vascular and lymphatic endothelial contamination and in conjunction with markers to exclude cells of leukocyte origin can be used to confirm the sinusoidal nature of the cells.

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EDITORIAL

Sporadic versus hereditary gastrinomas of the duodenum and pancreas: Distinct clinico-pathological and epidemiological features

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Abstract

Gastrinomas are defined as gastrin secreting tumors that are associated with Zollinger-Ellison syndrome (ZES). ZES is characterized by elevated fasting gastrin serum levels, positive secretin stimulation test and clinical symptoms such as recurrent peptic ulcer disease, gastroesophageal reflux disease and occasional diarrhea. Genetically, nonhereditary (sporadic) gastrinomas are distinguished from hereditary gastrinomas, which are associated with multiple endocrine neoplasia type 1 (MEN1) syndrome. In general, duodenal gastrinomas are small and solitary if they are sporadic and multiple as well as hereditary. The sporadic gastrinomas occur in the duodenum or in the pancreas while the hereditary gastrinomas almost all occur in the duodenum. Our series of 77 sporadic duodenal neuroendocrine tumors (NETs) includes 18 patients (23.4%) with gastrinomas and ZES. Of 535 sporadic NETs in the pancreas collected from the NET archives of the departments of pathology in Zürich, Switzerland, and Kiel, Germany, 24 patients (4.5%) suffered from sporadic pancreatic gastrinomas and ZES. These NETs have to be distinguished from

tumors with immunohistochemical positivity for gastrin but without evidence of ZES. An additional 19 patients suffered from MEN1 and ZES. These patients showed exclusively duodenal gastrinomas, but not pancreatic gastrinomas. The prognosis of sporadic and MEN1-associated duodenal gastrinomas is better than that of pancreatic gastrinomas, since they progress slowly to liver metastasis. In summary, sporadic and MEN1-associated gastrinomas in the duodenum and pancreas show different clinico-pathological and genetic features. The incidence of sporadic duodenal gastrin-producing tumors is increasing, possibly due to optimized diagnostic procedures. In contrast, pancreatic MEN1-associated gastrinomas seem to be extremely rare. A considerable subset of tumors with immunohistochemical expression of gastrin but without evidence of ZES should be designated as functionally inactive NETs expressing gastrin, but not as gastrinomas.

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Key words: Endocrine tumor; Gastrinoma; Multiple endocrine neoplasia type 1; Precursor lesion; Zollinger-Ellison syndrome

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INTRODUCTION

Gastrinomas are defined as gastrin-producing tumors that are associated with Zollinger-Ellison syndrome (ZES) due to inappropriate gastrin secretion. ZES is characterized by elevated fasting gastrin serum levels, positive gastrin secretin stimulation test and clinical symptoms such as recurrent peptic ulcer disease, gastroesophageal reflux disease and occasional diarrhea^[1,2].

The first cases of ZES were described in 1955^[3].

One patient, a 36-year-old woman with severe recurrent ulcer disease and a family history strongly suggestive of multiple endocrine neoplasia type 1 (MEN1) background, was found to have several endocrine tumors, including microadenomas, in the pancreas. This case report already illustrates many of the issues that are still encountered in the diagnosis of gastrinoma. A closer look at the report by Zollinger and Ellison, on the basis of our current knowledge on gastrinomas, reveals that they were most likely dealing with a MEN1 patient. What is the reason for this assumption? Zollinger and Ellison described multiple endocrine tumors in the pancreas, which they thought were the cause of the ulcer syndrome, since their removal by a Whipple resection cured the patient. However, today we know that multiple gastrinomas virtually do not exist in the pancreas, but virtually always occur in the duodenum in the setting of MEN1. In this hereditary syndrome duodenal tumors producing gastrin are tiny and usually associated with multiple pancreatic tumors that do not produce gastrin, but may be large. In 1955, it was not possible to prove that the tumors produced gastrin. Firstly, gastrin still has to be isolated^[4,5], and secondly, immunohistochemistry for gastrin has not yet been invented. Therefore, it is a quite likely assumption that Drs. Zollinger and Ellison's patient suffered from a recurrent ulcer disease in the setting of MEN1 syndrome and had multiple small gastrinomas in the duodenum, which were removed together with non-gastrin producing endocrine tumors in the pancreas. While the tumors in the pancreas were easily noticed and described, the duodenal minigastrinomas probably escaped detection.

This review focuses on the clinical setting and morphological aspects of sporadic and MEN1-associated duodenal and pancreatic gastrinomas. In addition, the results of an analysis of epidemiology of sporadic and MEN1-associated duodenal and pancreatic gastrinomas in a large series of duodenal and pancreatic neuroendocrine tumors (NETs) from the Swiss and German NET archives are presented.

CLINICAL SETTING OF GASTRINOMAS

Between 60% and 75% of patients with ZES are found to have an isolated duodenal or pancreatic gastrinoma (sporadic ZES). In the remaining patients ZES is part of MEN1 syndrome and these patients usually exhibit multiple duodenal gastrinomas (hereditary gastrinoma)^[6-8]. The term pseudo-ZES (also called ZES type 1, as opposed to ZES type 2 caused by a gastrinoma) is coined for a syndrome with symptoms similar to ZES that appears to be caused by antral G-cell hyperfunction and hyperplasia^[9,10]. The fact that this syndrome has no longer been described in recent years raises questions of whether it exists at all. In rare cases the syndrome of recurrent and intractable peptic ulceration may be found in association with a pancreatic endocrine tumor that does not produce and secrete gastrin^[11]. The factor causing peptic ulceration in these patients has yet to be identified^[12].

Among the gastroenteropancreatic neuroendocrine tumors associated with hormonal syndrome, gastrinomas are second only in incidence to insulinomas and are

malignant in more than 60% of the cases. These tumors are classified as low grade malignant neoplasms, i.e. well differentiated neuroendocrine carcinomas. The peak incidence of gastrinomas lies between 40 and 50 years, children (5-15 years of age) are rarely affected^[1].

SPORADIC GASTRINOMA

Sporadic gastrinomas occur either in the pancreas or in the duodenum and are apparently solitary tumors. In the past, approximately 70%-80% of these gastrinomas were thought to occur in the pancreas, particularly in its head. Currently, gastrinomas are more frequently found in the duodenum.

In general, gastrinomas represent the only type of endocrinologically active duodenal NETs, while all other types of duodenal NET (i.e. functionally inactive NETs expressing somatostatin, serotonin or gastrin, gangliocytic paraganglioma and poorly differentiated neuroendocrine carcinomas) are found to be endocrinologically silent. The reason for the increasing incidence of sporadic duodenal gastrinomas and endocrinologically silent inactive gastrin-producing NETs may be that many of these small NETs were overlooked but their large periduodenal/peripancreatic lymph node metastases were noted and recorded as primary gastrinoma in the pancreas or as primary lymph node gastrinoma in the past (details are shown below).

Sporadic duodenal gastrinomas usually arise from the first part of the duodenum and are located in the submucosa. They are most often less than 1 cm in diameter^[13-16] (Figure 1A and 1B). Despite this small size metastases to regional lymph nodes are already found in 60% to 80% of the patients at the time of diagnosis^[13]. It seems that periduodenal and peripancreatic lymph node metastases may grow faster than their duodenal primary tumors and thus may form large tumors that are easily recognized, in contrast to the duodenal primary tumors. It has therefore been suggested that the so-called peripancreatic and periduodenal lymph node gastrinomas that were described in the past may in fact be metastases of duodenal microgastrinomas that are overlooked during diagnostic work-up and surgery, rather than true primary tumors^[17-19]. Apart from lymph node metastases, duodenal gastrinomas may metastasize to the liver, but only in a small percentage of cases (about 10%) and only many years after the manifestation of the disease^[13]. Thus the 10-year survival rate of 84% has been reported in patients with duodenal gastrinomas^[20,21]. Fast growing and metastasizing duodenal gastrinomas are rare.

Histologically, duodenal gastrinomas are often submucosal tumors that infiltrate the mucosa and may also infiltrate the muscular layer if they are larger than 1 cm in diameter. They most often show a trabecular or pseudoglandular pattern. Their proliferative activity is usually between 2% and 10% (Figure 2). Some of the tumors may show angioinvasion. Their prognostic classification is outlined in detail in Table 1. Immunocytochemically, gastrin can be detected in all tumors^[7,22]. Many duodenal gastrinomas are multihormonal and additionally contain single somatostatin or serotonin

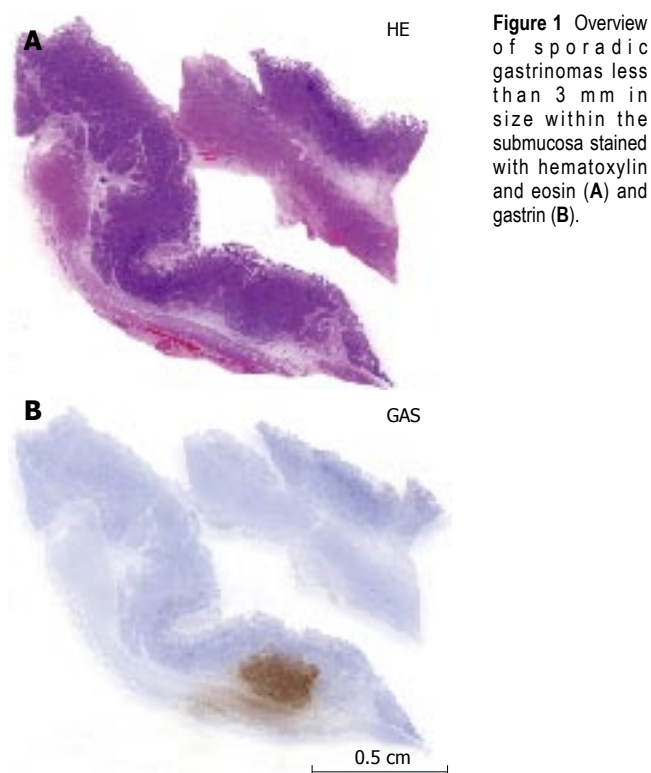


Figure 1 Overview of sporadic gastrinomas less than 3 mm in size within the submucosa stained with hematoxylin and eosin (A) and gastrin (B).

expressing cells in addition to gastrin cells.

Sporadic gastrinomas in the pancreas usually have a diameter of 2 cm or more (Figure 3). It has been reported that they occur more frequently in the head of the pancreas^[13]. However, in our series they were found in all parts of the organ.

Metastasis of sporadic pancreatic gastrinomas to regional lymph nodes is found in approximately 60% of patients at the time of diagnosis^[23], and liver metastases occur more frequently (10%-20%) than duodenal gastrinoma liver metastasis^[17,18,23]. Thus the 10-year survival rate is worse in patients with pancreatic gastrinomas (57%) than in patients with duodenal gastrinomas (84%)^[20,21]. In rare cases, bone metastases may develop in the terminal phase of a metastasized gastrinoma.

Histologically, pancreatic gastrinomas are similar to duodenal gastrinomas, but may have a higher proliferation and angioinvasion rate. Table 1 shows their prognostic assessment. Immunocytochemically, gastrin can be detected in almost all tumors^[7,22]. Approximately 50% of gastrinomas are multihormonal and contain PP, glucagon and/or insulin in addition to gastrin.

Islet hyperplasia and nesidioblastosis have repeatedly been described in the non-neoplastic pancreas of patients with gastrinomas, but these findings cannot be confirmed by morphometry^[24]. Recently, however, morphometrically defined PP-cell hyperplasia has been described in the ventrally derived region of the pancreatic head^[25]. It has not been definitely established whether hypergastrinemia can influence these changes. In the stomach mucosa, however, sustained hypergastrinemia induces parietal cell hyperplasia with thickened mucosal folds and gastric acid hypersecretion. In addition, the number of enterochromaffin-like (ECL) cells is increased

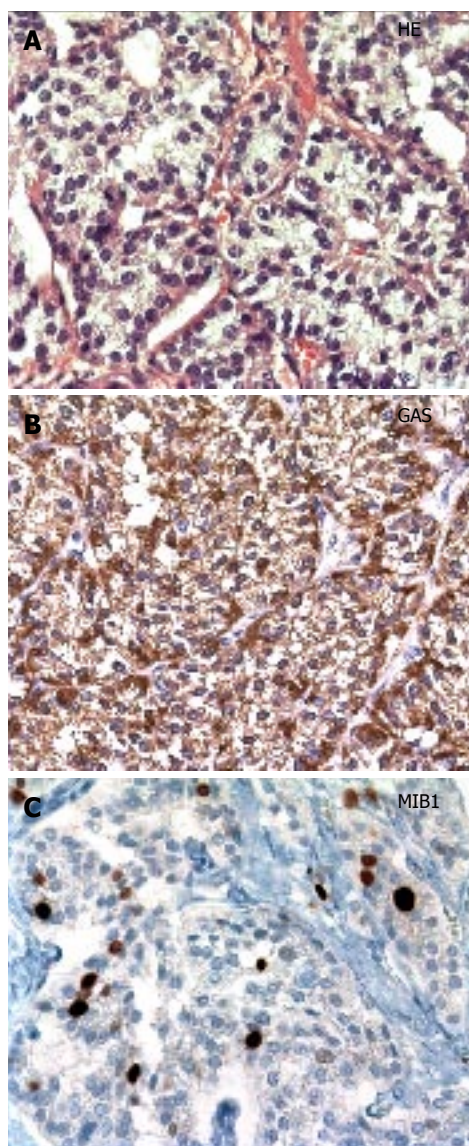


Figure 2 Morphology of sporadic duodenal gastrinoma stained with HE showing a trabecular and glandular growth pattern (A); strong immunoreactivity for gastrin (B), and expression of the nuclear proliferation antigen MIB1 in more than 2% of NET cells (C).

in the fundal mucosa^[26-28]. ECL cell tumors in the fundus of the stomach, which are a well-known complication in patients suffering from pernicious anemia due to chronic type A gastritis, appear to be very uncommon in patients with sporadic ZES^[7]. They have, however, been reported in patients with ZES and MEN1. In these instances they probably represent another neoplastic manifestation of MEN1 syndrome (see below) rather than merely the result of a trophic effect of gastrin^[26,29].

MEN1-ASSOCIATED GASTRINOMAS

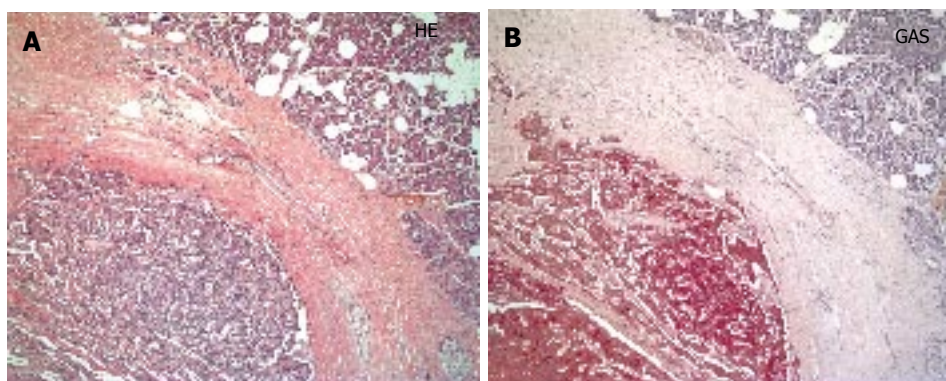
Approximately 25%-33% of patients with gastrinomas develop these tumors in the setting of MEN1. Almost all these gastrinomas reside in the duodenum^[30]. They are usually smaller than 1 cm in diameter and multicentric, arising from multifocal hyperplastic gastrin cell proliferations^[31] (Figures 4 and 5). Histologically,

Table 1 Classification of neuroendocrine tumors of the pancreas (WHO classification 2004)^[41]

1	Well-differentiated neuroendocrine tumor
	<ul style="list-style-type: none"> Benign: confined to pancreas, < 2 cm in size, nonangioinvasive, ≤ 2 mitoses/HPF and ≤ 2% Ki-67-positive cells <ul style="list-style-type: none"> - Functioning: insulinoma - Nonfunctioning Benign or low grade malignant (uncertain malignant potential): confined to pancreas, ≥ 2 cm in size, > 2 mitoses/HPF, > 2% Ki-67-positive cells, or angioinvasive <ul style="list-style-type: none"> - Functioning: gastrinoma, insulinoma, VIPoma, glucagonoma, somatostatinoma, or ectopic hormonal syndrome - Nonfunctioning
2	Well-differentiated neuroendocrine carcinoma
	<ul style="list-style-type: none"> Low grade malignant: invasion of adjacent organs and/or metastases <ul style="list-style-type: none"> - Functioning: gastrinoma, insulinoma, glucagonoma, VIPoma, somatostatinoma or ectopic hormonal syndrome - Nonfunctioning
3	Poorly-differentiated neuroendocrine carcinoma
	<ul style="list-style-type: none"> High grade malignant

Table 2 Classification of neuroendocrine tumors of the duodenum and upper jejunum

1	Well-differentiated neuroendocrine tumor
	<ul style="list-style-type: none"> Benign: nonfunctioning, confined to mucosa-submucosa, nonangioinvasive, ≤ 1 cm in size <ul style="list-style-type: none"> - Gastrin-producing tumor (upper part of the duodenum) - Serotonin-producing tumor - Gangliocytic paraganglioma (any size and extension, periampullary) Benign or low grade malignant (uncertain malignant potential): confined to mucosa-submucosa, with or without angioinvasion, or > 1 cm in size <ul style="list-style-type: none"> - Functioning gastrin-producing tumor (gastrinoma), sporadic or MEN-1 associated - Nonfunctioning somatostatin-producing tumor (ampullary region) with or without neurofibromatosis type 1 - Nonfunctioning serotonin-producing tumor
2	Well-differentiated neuroendocrine carcinoma
	<ul style="list-style-type: none"> Low grade malignant: invasion of the muscularis propria and beyond or metastases <ul style="list-style-type: none"> - Functioning gastrin-producing carcinoma (gastrinoma), sporadic or MEN-1 associated - Nonfunctioning somatostatin-producing carcinoma (ampullary region) with or without neurofibromatosis type 1 - Nonfunctioning or functioning carcinoma (with carcinoid syndrome) - Malignant gangliocytic paraganglioma
3	Poorly-differentiated neuroendocrine carcinoma
	<ul style="list-style-type: none"> High grade malignant

**Figure 3** Overview of sporadic pancreatic gastrinoma surrounded by thickened collagen in the vicinity of normal pancreatic parenchyma stained with hematoxylin and eosin (A) and gastrin (B).

they show trabecular and pseudoglandular patterns and immunohistochemically they express gastrin and occasionally also somatostatin. Because of their small size they are (like sporadic duodenal gastrinomas) difficult to detect. Pancreatic gastrinomas associated with MEN1 are very rare^[6,32], although the pancreas of these patients usually contains multiple endocrine micro- and macrotumors^[33]. These tumors, however, virtually never produce significant amounts of gastrin^[6,32]. The metastatic and biological behavior of duodenal MEN1-associated

gastrinomas is similar to that of sporadic counterparts (Table 2).

EXTRADUODENAL AND EXTRAPANCREATIC GASTRINOMAS

Unusual sites of gastrinomas are the stomach^[34], jejunum^[35,36], biliary tract, liver^[37] and kidney^[38]. Ovarian or pancreatic mucinous cystic tumors that contain a sufficient number of active endocrine cells with gastrin production

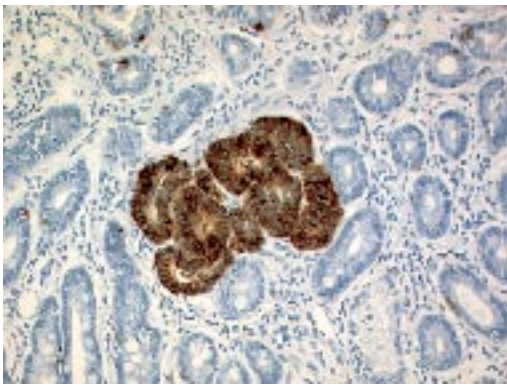


Figure 4 Circumscribed linear and nodular hyperplasia of gastrin cells within the Brunner's glands in a patient with MEN1.

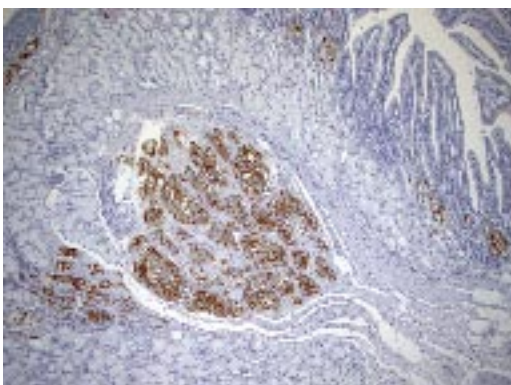


Figure 5 Tiny MEN1-associated duodenal gastrinoma within the submucosa revealing a diameter of less than 1 mm.

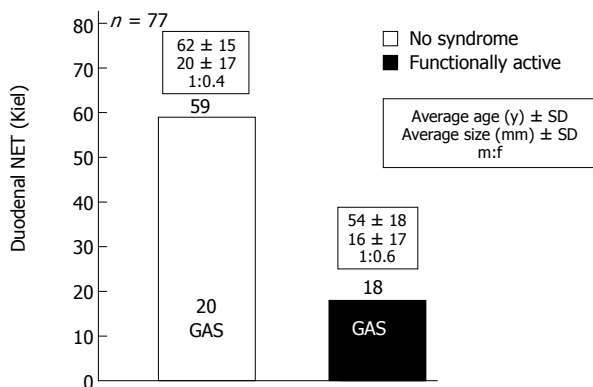


Figure 6 Duodenal NETs in the Kiel tumor archive. Fifty-nine (76.6%) out of the 77 NETs were endocrinologically not active, 20 of them expressed gastrin. These were not associated with ZES. All the functionally active NETs (18; 23.4%) were immunohistochemically positive for gastrin and showed a ZES.

may also cause ZES, but are uncommon^[39-41].

PERSONAL OBSERVATIONS

In our series of sporadic duodenal NETs collected from 1975 to 2006, duodenal gastrin producing tumors account for 49.4% (38) of 77 sporadic NETs (Figure 6). Surprisingly, only 47.4% (18) of the gastrin-immunoreactive sporadic NETs show an association

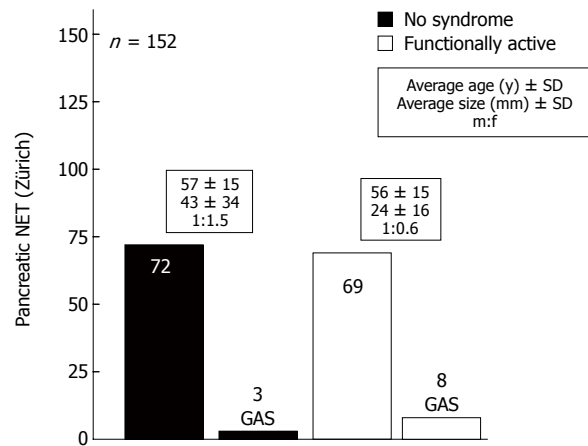


Figure 7 Pancreatic NETs in the Zürich tumor archives. Seventy-five (49.3%) out of the 152 pancreatic NETs were endocrinologically not active, of which 3 expressed gastrin. These 3 gastrin-expressing NETs were not associated with ZES. Eight of the 77 functionally active NETs were immunohistochemically positive for gastrin and associated with ZES.

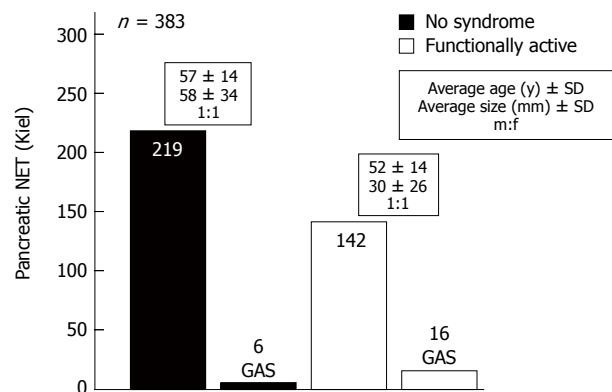


Figure 8 Pancreatic NETs in the Kiel tumor archives. Two hundred and twenty-five (58.7%) out of the 383 pancreatic NETs were endocrinologically not active. These gastrin-expressing NET were not associated with ZES. Sixteen of the 158 functionally active NETs were immunohistochemically positive for gastrin and showed a ZES.

with ZES (Figure 6). The reason for the lack of ZES in a considerable subset of patients with gastrin-expressing tumors remains to be analyzed in detail. Whether these gastrin producing tumors in the duodenum are similar in behavior to the duodenal gastrinomas remains unknown. However, it seems that they may have a different biology. Terminologically, these NETs with immunohistochemical expression of gastrin but without evidence of ZES should be designated as functionally inactive NETs expressing gastrin, but not as gastrinomas.

In two large series of sporadic pancreatic NETs from Kiel ($n = 383$) and Zürich ($n = 152$) pancreatic gastrinomas were found to be rare tumors, accounting for 4.2% (Kiel) and 5.3% (Zürich) of all collected sporadic tumors, respectively. Similar to duodenal tumors an additional 1.6% (Kiel) and 2.0% (Zürich) of sporadic gastrin-expressing tumors were not associated with ZES and were therefore designated as functionally inactive pancreatic NETs producing gastrin (Figures 7 and 8).

It was reported that 19 (59.4%) of 32 patients with MEN1 showed ZES. The source of ZES in these patients

is duodenal rather than pancreatic gastrinomas. Most of these exhibit multifocal duodenal gastrinomas and lymph node metastases^[31,33].

CONCLUSION

The preferred site of gastrinomas is the duodenum rather than the pancreas. Despite the small size of duodenal gastrinomas they may show the same rate of metastasis at the time of diagnosis as pancreatic gastrinomas, which are usually larger in size. However, the survival rate of patients with pancreatic gastrinomas is lower than that of patients with duodenal gastrinomas. MEN1-associated gastrinomas are virtually all localized in the duodenum. They are usually multiple. They probably arise from multifocal precursor lesions, i.e. diffuse gastrin cell proliferations that are lacking in sporadic duodenal gastrinomas. Biologically, the behavior of MEN1-associated gastrinomas is similar to that of sporadic duodenal gastrinomas. Gastrin expressing tumors both in the duodenum and in the pancreas without evidence of ZES should be designated as functionally inactive NETs producing gastrin, but not as gastrinomas. The reasons for the lack of hormonal symptoms in gastrin expressing NETs still need to be analyzed in detail.

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Do probiotics have a therapeutic role in gastroenterology?

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Abstract

Several hundred species of bacteria inhabit the gut, and affect its cell biology, morphology and homeostasis. Many bacteria are however potential pathogens, especially if the integrity of the epithelial barrier is physically or functionally breached. Conversely, the interaction between host and commensal microbes can confer important health benefits. This has led to commercial and public interest in 'probiotics', live microbes principally taken as food supplements. Might probiotics also be used in disease therapy? Experimental evidence that probiotics modulate gut physiology, particularly barrier integrity and immunological function, underpins exciting new gastroenterological research. We discuss below the scientific basis for probiotic effects and present a critical perspective for their use in relation to gastrointestinal disease.

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Key words: Probiotics; Gastroenterology

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INTRODUCTION

The concept of probiotics probably dates back to 1908, when Nobel Prize winner Eli Metchnikoff suggested that the long life of Bulgarian peasants resulted from their consumption of fermented milk products^[1]. In 1965 Lilly and Stillwell first used the term 'probiotic' when describing 'substances secreted by one organism which stimulate the growth of another'^[2]. Parker^[3] described probiotics as

'organisms and substances which contribute to intestinal microbial balance' and Fuller proposed in 1989 that probiotics were 'a live microbial supplement which beneficially affects the host animal by improving its microbial balance'^[4]. Salminen *et al*^[5] defined them as 'foods containing live bacteria which are beneficial to health', whilst Marteau *et al*^[6] define them as 'microbial preparations or components of microbial cells that have a beneficial effect to health and well being'. Such definitions underpin the current popular commercial usage of various 'friendly bacteria' to secure non-specific benefits to health.

With improved understanding of the physiology and therapeutic role of probiotics, definitions have evolved bolder claims, which now enter medical territory. Charteris *et al*^[7] defined probiotics as 'micro-organisms, which when ingested, may have a positive effect in the prevention and treatment of a specific pathological condition'.

Two related terms are prebiotics and synbiotics: *prebiotics* are defined as "non digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve host health"^[8]. When prebiotics and probiotics are administered together, this is referred to as a synbiotic.

NORMAL GUT MICROFLORA

The colon contains tenfold more bacteria than the total number of mammalian cells constituting the host. The relationship is symbiotic: for example, bacterial vitamin K synthesis contributes to haemostasis, whilst short chain fatty acids generated by colonic bacteria salvage additional energy from otherwise 'wasted' dietary fibre.

Colonisation of the gastrointestinal tract starts immediately after birth, initially with maternal vaginal and intestinal flora. Other sources are diet (breast or formula based feeds)^[9-12] and environment, as reflected by the different gut flora in infants born in developing and developed countries^[13-15]. Infants who are breast fed predominantly harbour *Bifidobacteria* whilst formula fed infants have a more complex bacterial profile comprising *Enterobacteria*, *Bacteroides*, *Clostridia*, *Lactobacilli*, *Bifidobacteria* and *Streptococci*^[11].

These 'pioneer' bacteria are important because they modulate epithelial cell gene expression, creating a favourable habitat for themselves by inhibiting the growth of bacteria introduced later^[16]. This renders initial colonisation causally determinant to the final composition of bacterial flora in adults^[17].

During development, the gut flora changes. The mouth

harbours mainly facultative and strict anaerobes including *Streptococci*, *Bacteroides* and yeasts. The oesophagus has no significant resident microbial colonisation but is constantly transited by swallowed organisms. The widely held idea that the upper gut is largely sterile is not valid. The stomach and duodenum harbour up to 10^4 colony forming units (CFU) per gm of *Candida albicans*, *Bacteroides*, *Lactobacillus* and *Streptococcus*. *H. pylori* are specifically adapted for gastric residence. The jejunum again harbours *Bacteroides*, *Candida albicans*, *Lactobacillus* and *Streptococcus* but with a content of 10^5 - 10^7 CFU/g. Scepticism that probiotics will not survive the passage through the acidic stomach is therefore unfounded. From the ileum onwards bacterial colonisation increases from 10^7 - 10^8 CFU/g in the ileum to 10^{10} - 10^{11} CFU/g in the colon, with a predominance of *Bacteroides*, *Bacillus*, *Clostridium*, *Enterococcus*, *Peptostreptococcus*, and *Streptococcus* species^[18].

GUT MICROFLORA AND GUT PHYSIOLOGY

Recent research has demonstrated that a dynamic and reciprocal interplay exists between the gut microflora and the host. In particular, there is growing evidence that bacteria play an important role in directing epithelial differentiation and reinforcement of the gut barrier, through complex host-bacterial cross talk which occurs at a molecular level in interacting cells. This new biology underpins the putative effects of probiotics, since host-bacterial interactions can be re-engineered by purposeful manipulation of luminal ecology.

The physical barrier: bacteria and tight junctions

The intestinal epithelium constitutes an anatomical and functional barrier, effectively a bipolar monocellular obstacle between luminal microbes and the cells of the lamina propria. Barrier function is normally maintained by a complex interplay of numerous proteins, assembled to form the tight junction complexes (TJs)^[19,20] in the juxta-apical region of the cell membrane (Figure 1). Commensal organisms contribute uncharacterised constitutive signals supporting epithelial integrity. Disruption of TJs may be elicited by pathogenic bacteria, stress and injury, *via* pro-inflammatory cytokines. Disruption of TJ integrity results in increased paracellular permeability, which is measurable in a variety of experimental models, and which may initiate, exacerbate or perpetuate intestinal inflammation in disease^[21]. Altered intestinal permeability has been demonstrated in Crohn's disease, celiac disease, intestinal infections and NSAID-induced enteropathy^[22]. In order to counteract the harmful effects of luminal pathogens and toxins, and to protect barrier homeostasis, intestinal epithelial cells exhibit several additional defensive features, which include production of defence peptides and mucins^[23]. In addition, a class of bacterial-sensing immunocytes, the dendritic cells, are able to project sensory dendrites into the lumen between adjacent enterocytes *via* TJ regions. Dendritic cells express receptors evolved to sense bacterial components, through which highly patterned host immune responses are evoked appropriately and constantly. Supporting experiments in rats have shown

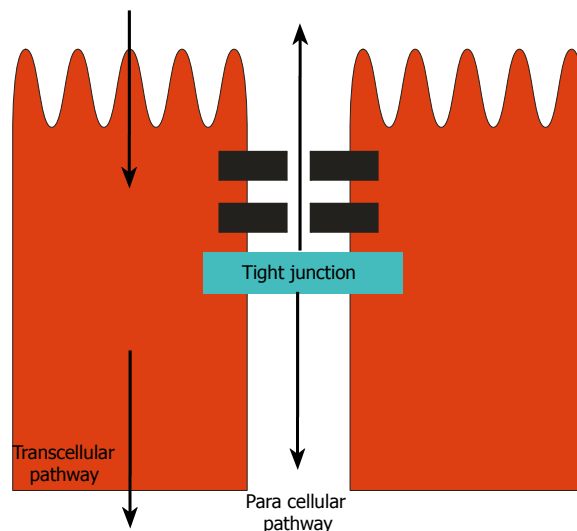


Figure 1 Tight junctions have a 'barrier' function in epithelia.

that colonisation of an excluded colonic loop with *E. coli* increased paracellular permeability, but this was partially reversed by colonisation with a putative probiotic, *Lactobacillus brevis*^[24]. In addition, the chronic colitis occurring in Interleukin-10 (IL-10) deficient mice^[25] is associated with increased colonic permeability, which is reversed in mice pre-treated for 4 wk. with a probiotic product, VSL#3 consisting of *Bifidobacterium*, *Streptococcus* and *Lactobacillus* species.

The potential cellular basis has also been addressed in human cell models^[26]. The influence of whole probiotic bacteria *E. coli* Nissle 1917 (EcN) or VSL#3, bacterial cell lysates or conditioned medium from bacterial cultures were assessed against a variety of barrier and defensive parameters in human intestinal epithelial cell lines. These included a measure of TJ function (transepithelial resistance, TER) and tight junction protein abundance, IL-8 secretion and mucin gene expression. In addition, protective effects on pathogen (*Salmonella dublin*) induced alterations were analyzed. The probiotic mixture and soluble protein released from it, increased basal TER, prevented pathogen-induced decrease in TER, and stabilized TJs. This suggested that the organisms, or their secretory products, functionally modulate the intestinal epithelium of the host. These include competition of organisms for contact with the epithelial surface, stabilization of the cytoskeletal and barrier function, and the induction of mucin gene expression. Gram-negative and gram-positive organisms differed in the cellular mechanisms activated: perhaps a combination of organisms might be more effective than the application of a single strain^[26]. The stumbling block inevitably lies in translating observations in these reductionist models to a proven clinical effect.

Recent interest has centred on regulation of the barrier *via* epithelial sensing of microbial components. The Toll-like receptors (TLRs) are a class of pattern-recognition receptors that specifically discriminate between self and microbial non-self based on the recognition of molecular patterns^[27]. TLRs thereby play an important role in im-

immune responses and the induction of antimicrobial effector pathways, leading to elimination and exclusion of host-threatening pathogens. It has been shown that the intestinal epithelium expresses several TLRs, which include TLR2, TLR3, TLR4 and TLR5^[24-34]. It is possible that certain probiotic agents contain TLR-specific immunostimulatory features, leading to the amelioration of colitis by restoring intestinal epithelial barrier to protect the host^[35].

The functional barrier: probiotics and mucosal immunity

The gastrointestinal mucosa is the primary interface between the external environment and the immune system. In the complete absence of intestinal microflora antigen transport is increased, indicating that the normal gut microflora maintain gut defences^[25]. The microflora affect the development of gut associated lymphoid tissue at an early age by directing the regulation of systemic and local immune responsiveness, including promoting tolerance via hypo-responsiveness to antigens from micro-organisms and food^[36]. Probiotic organisms have been shown to modulate immunoglobulin production. Secretory IgA plays an important role in mucosal immunity, contributing functionally to the barrier against pathogenic bacteria and viruses^[37-40]. An enhanced IgA immune response has been shown in children with Crohn's disease treated with *Lactobacillus* GG^[41]. Interestingly, Madsen *et al.*^[42] demonstrated that IL10 deficient mice displayed significantly higher basal numbers of adherent bacteria compared with healthy control mice. When the colon was repopulated with *Lactobacillus reuteri* enemas the proportion of adherent and translocated bacteria, and the development of colitis, was significantly decreased. Further, Schultz^[43] demonstrated that feeding *Lactobacillus plantarum* attenuated established colitis in IL-10 knockout mice. In rats, the effects of *Lactobacilli* and fibre (oatbase) were studied in Methotrexate-induced enterocolitis. Rats received an intragastric infusion of an elemental diet, with or without supplementation of oatbase, *Lactobacillus reuteri* R2LC and *Lactobacillus plantarum* DSM9843^[44]. Methotrexate was injected intraperitoneally on d 3. By d 6 *Lactobacillus* decreased intestinal inflammation, re-established intestinal microecology and reduced bacterial translocation to extra-intestinal sites.

Data from human studies support a role for the gut microflora in the development of several gut associated inflammatory conditions, most likely triggered by immune response to their antigenic structures^[45]. Thus probiotics may exert clinical effects by altering the intestinal inflammatory response to the luminal microflora.

Trophic and nutritional effects of gut microflora

Probiotic organisms exert a potentially positive effect on gut function through a trophic action on gut mucosa. In experimental models, crypt cell turnover is reduced in the colon of rats bred in germ free environments (gnotobiotic animals). Germ free crypts also contain fewer cells than those of colonised rats^[46]. Butyrate is an important source of energy for colonocytes^[47], whilst acetate and propionate are found in portal blood and are eventually metabolised in the liver or peripheral tissues, in particular muscle^[47,48]. The most important role of SCFA is probably their trophic

effect on colonic epithelium. Short chain fatty acids stimulate epithelial cell proliferation and differentiation in large and small bowel *in vivo*^[49]. Butyrate however inhibits cell proliferation in epithelial cell lines of neoplastic origin^[50]. Further, butyrate is pro-apoptotic and promotes reversion of cells from neoplastic to non-neoplastic phenotypes^[51]. SCFA generation can clearly be altered by manipulating colonic micro-ecology, so probiotics and prebiotics may find a role in the prevention of colonic neoplasia and in the therapy of inflammatory bowel diseases.

Colonic micro-organisms play an important role in vitamin synthesis^[52,53] and in the absorption of calcium, magnesium and iron^[54-56]. Ion absorption in the caecum is improved by carbohydrate, and production of short chain fatty acids particularly acetate, propionate, and butyrate.

Interactions with mucus

A mucus gel covers the gut epithelium acting as a protective barrier against pathogens and reducing physical trauma. Change in the mucus content or structure compromise barrier function. Interactions occur between bacteria and mucus, including the probiotic bacteria that bind to intestinal mucus^[57]. This is potentially advantageous since it inhibits adhesion of enteropathogenic bacteria to mucus. For example *Enterococcus faecium* inhibits the adhesion of enterotoxigenic *E. coli* K88 to porcine small intestine mucus^[58].

PROBIOTICS IN CLINICAL GASTROENTEROLOGY

The experimental data discussed above demonstrate that several 'probiotic' organisms exert biological effects which might translate into clinical benefits. Current clinical evidence is limited and non-uniform. Key observations in relevant conditions are discussed below.

Probiotics in GI infections

Probiotics have an emerging role in the treatment of gastrointestinal infections. Probably the best described is in acute infantile diarrhoea. *Lactobacillus* strain GG in fermented milk or freeze-dried powder was shown to reduce the duration of diarrhoea in acute rotavirus infection compared to a placebo group given pasteurised yoghurt^[59]. Other studies have confirmed these results^[60,61]. Suggested mechanisms of action are stabilisation of indigenous microflora^[62], reduction in the increased gut permeability caused by rotavirus infection^[63] and reduction in the duration of virus shedding^[64]. This may be cause-specific. In a multicentre European trial of probiotics in acute childhood diarrhoea caused by rotavirus and other pathogens^[65], probiotics (*Lactobacillus* GG) shortened the duration of diarrhoea in rotavirus diarrhoea, but showed no such effect with other pathogens.

Probiotics may also have a role in the prevention of acute infantile diarrhoea. In a double blind, placebo controlled trial hospitalised infants were randomised to receive a standard infant formula alone or supplemented with *Bifidobacterium bifidum* and *Streptococcus thermophilus*. After a 17-mo follow up period, 7% of those receiving a probiotic

had experienced diarrhoea compared to 31% given the standard formula^[66]. Viral shedding was lower in the probiotic supplemented group. Prophylactic use of *Lactobacillus* GG in 204 undernourished children followed up for 15 mo also decreased the incidence of acute diarrhoea^[67]. This effect was confined to non-breast fed infants. In a more recently reported double blind randomised placebo controlled trial, 81 children between 1-3 years of age, hospitalised for reasons other than diarrhoea, and were given *Lactobacillus* GG or placebo for the duration of their admission. The incidence of nosocomial diarrhoea was lower in the probiotic group (7% as compared to placebo, 33%)^[68]. Furthermore, although the prevalence of rotavirus infection was similar in both groups, the risk of rotavirus gastroenteritis was lower in the probiotic group. Finally, the benefits of *Lactobacillus* GG in rotavirus associated diarrhoea mainly comprise a reduction in duration of diarrhoea by 1-2 d compared to a median of 3 d.

The positive results for *Lactobacillus* GG in acute viral diarrhoea cannot necessarily be extrapolated to other probiotic strains, nor to other causes of acute diarrhoea. The data imply species and disease specificity. For example, in a study comparing various probiotic strains, *Lactobacillus* GG was found to have a beneficial effect in rotavirus gastroenteritis but this was not shared by *L. rhamnosus*, *L. delbrueckii* or *Streptococcus thermophilus*^[60]. *Saccharomyces boulardii* has also displayed beneficial effects in acute diarrhoea in children and adults^[60,70]. Enterococcus SF68 in adults with acute diarrhoea has however shown inconsistent results^[71-73]. It is unclear whether the modest benefits suggested by these studies justify routine use of probiotics in diarrheal illnesses since most acute diarrhoeal diseases are self limited. The benefit may be greatest in situations where patients are at risk for complications such as in children with malnutrition in developing countries.

Antibiotic-associated diarrhoea

Diarrhoea can occur as an adverse effect of antibiotic therapy, in up to 39% of antibiotic treated hospitalised patients^[74]. Broad spectrum antibiotics are more commonly implicated, possibly because of more profound alteration in colonic flora^[75]. Several placebo-controlled studies have shown a decrease in the incidence of diarrhoea or change in stool consistency when patients were treated with probiotics in addition to antibiotics^[76-82]. The probiotic organisms studied were *Lactobacillus* spp., Enterococcus and *Saccharomyces boulardii*. Not all studies support this possibility. A recent study of 267 patients on antibiotics randomised to *Lactobacillus* GG or placebo failed to show any decrease in incidence of diarrhoea, with 29% in both groups developing symptoms^[83]. Three other smaller studies were also negative^[78,84,85]. Although most studies looking at probiotics in antibiotic-associated diarrhoea are placebo controlled and conducted on a reasonable number of subjects, different antibiotics were used in these studies contributing to their heterogeneity. Two recent meta-analyses (of nine and seven randomised placebo controlled double blind trials) have looked at the effect of probiotics in prevention of antibiotic-associated diarrhoea^[86,87]. The meta-analysis by D'Souza *et al*^[87] reviewed nine randomised, double blind, placebo controlled trials. Four trials used yeast (*Saccharomyces*

boulardii); four used *Lactobacilli* and another used a strain of enterococcus that produced lactic acid. Three trials used a combination of probiotic bacteria. Antibiotics were given with probiotics (or with placebo, in the control group) in all nine trials. The combined odds ratio in favour of active treatment over placebo in preventing diarrhoea associated with antibiotics was 0.37 (95% confidence interval, 0.26 to 0.53; $P < 0.001$). In the meta-analysis by Cremonini *et al*^[86], twenty two studies using *Lactobacillus* and *Saccharomyces* species, matched the inclusion criteria of which seven studies were homogenous. The combined relative risk was 0.399 (95% confidence interval, 0.27-0.57) suggesting a strong benefit of probiotic administration on antibiotic associated diarrhoea. The pooled results suggested that probiotic administration had an overall benefit. However, the published data is discordant in that it is unclear what the optimal dose and timing of supplementation should be.

Clostridium difficile associated diarrhoea (CDAD)

Clostridium difficile is a Gram positive bacterium that can cause colitis, mediated by two enterotoxins, enterotoxin A and B. The pathophysiology is not fully clear but risk factors include intercurrent or continued antibiotic therapy, elderly age, renal disease and female sex^[88,89]. Probiotics have been partially evaluated in the prevention of CDAD. Early uncontrolled trials using *Lactobacillus* GG^[90-92] and a preliminary report of a controlled trial using *Lactobacillus* GG suggested benefit in recurrent CDAD^[93]. Similarly uncontrolled or open label studies^[94,95] and subsequently two controlled trials^[96,97] have suggested efficacy of *Saccharomyces boulardii* in recurrent CDAD. In the study by McFarland *et al*^[94], 124 patients were studied, including 64 patients with an initial episode of CDAD and 60 who had at least one previous episode of CDAD. Subjects received oral *Saccharomyces boulardii* (1 g/d for 4 wk or placebo) and were followed up for an additional 4 wk after therapy. Multivariate analysis showed that patients treated with *S. boulardii* and standard antibiotics had a significantly lower risk of CDAD (relative risk 0.43; 95% confidence interval, 0.20-0.97) compared with placebo. In their subsequent study, Surawicz *et al*^[97] tested patients receiving a standard antibiotic for 10 d and then added *S. boulardii* (1 g/d for 28 d) or placebo. A significant decrease in recurrence of CDAD (16.7%) was observed in patients treated with high-dose Vancomycin (2 g/d) compared with those receiving Vancomycin and placebo (50%; $P < 0.05$). Most studies were small and were mostly not placebo controlled.

A recent systematic review looking at studies in which probiotic therapy was used for prevention and treatment of *C. difficile*- associated diarrhoea concluded that the evidence for routine clinical use of probiotics in this setting was insufficient^[98]. Again, although probiotics are generally considered safe, case reports have described *Saccharomyces cerevisiae* fungaemia and deaths in immunocompromised and critically ill patients who received a commercial preparation of *S. boulardii* (genomically identical to *S. cerevisiae*)^[99]. Their routine use can therefore not be recommended.

Probiotics in Inflammatory Bowel Diseases

Although the etiology of inflammatory bowel diseases is

not entirely clear, dysfunctions in both innate and acquired immunity are implicated. There has been an increased interest in pathogenic and endogenous intestinal flora, with supportive data derived from several animal models. As noted above spontaneous colitis may develop in mice deficient in the immunoregulatory cytokine IL-10 but IL-10 deficient germ free mice remain disease free^[100-102].

Clinical studies suggest a significant role for bacteria in the pathogenesis of human IBD. Crohn's disease activity has been shown to improve with antimicrobial therapy, faecal diversion^[103,104], bowel rest and intestinal lavage^[105]. Furthermore, antibiotics may reduce postoperative relapse^[106], postoperative pouchitis^[107] and fistula related complications^[108]. There has been particular interest recently in polymorphisms in the CARD15/NOD2 gene, an intracellular bacterial pattern recognition receptor, as a risk factor for the development of Crohn's disease^[109]. Probiotics may therefore exert benefits in IBD management by modulatory effects on intestinal flora. For example *Lactobacillus* GG, when administered to children with Crohn's disease, increased mucosal IgA levels^[41] improved intestinal permeability and reduced disease activity^[110]. Further, the relapse rate in 32 adults with inactive Crohn's disease was reduced to 6% when subjects in remission were treated with mesalazine and *S. boulardii* as compared to 38% with mesalazine alone^[111]. However, in a placebo controlled study of 37 Crohn's disease patients treated with *Lactobacillus* GG for 12 mo after curative resection, the probiotic did not prevent relapse: in fact more severe endoscopic findings were reported in the *Lactobacillus* group^[112].

Recent clinical studies have evaluated the effect of non-pathogenic *E. coli* strain Nissle 1917 versus mesalazine for maintenance of remission in ulcerative colitis. Kruis *et al*^[113] studied 120 and Rembacken *et al*^[114] 116 patients with inactive ulcerative colitis over a period of 12 wk and 1 year respectively given either *E. coli* strain Nissle 1917 (MutaflorR) or mesalazine. These unblinded studies found a similar relapse in both groups (73% in the mesalazine group and 67% in the *E. coli* group), suggesting that probiotic therapy may be an alternative maintenance therapy. Similar results were suggested in a third RCT study^[115]. 327 patients were treated with mesalazine or *E. coli* Nissle 1917 for twelve months. Relapse rates were similar (45.1% in the probiotic group versus 36.4% in the mesalazine group). In a controlled trial by Tursi *et al*^[116], low dose balsalazide with VSL#3 was shown to be more effective than balsalazide or mesalazine alone in patients with acute mild to moderate ulcerative colitis. The combination of a prebiotic and a probiotic (*Bifidobacterium longum*/Synergy 1) was associated with improvement in histological scores and measures of immune activation in a randomised controlled pilot study^[117].

A recent study investigated the expression and function of CARD15/NOD2 in intestinal epithelial cell lines. CARD15/NOD2 mRNA was expressed in both intestinal epithelial cell lines and primary intestinal epithelial cells. CARD15/NOD2 mRNA and protein were up-regulated by tumor necrosis factor alpha (TNF alpha) in SW480 cells. This study suggests that CARD15/NOD2 may serve as a key component of innate mucosal responses to luminal bacteria as an antibacterial factor^[109]. Failure in this acti-

vity may contribute to the development of Crohn's disease.

Probiotics in pouchitis

Pouchitis is an inflammation of an ileal reservoir surgically constructed in the management of IBD. It is associated with reduced counts of *Bifidobacteria* and *Lactobacilli* with increased numbers of *Clostridia* and anaerobes in faecal samples^[118]. Increases in bile acids and decreases in short chain fatty acids, with a net increase in pH, may also be seen^[118]. In a double blind randomized placebo controlled trial a probiotic mixture VSL#3 was studied over 9 mo in 40 patients with chronic relapsing pouchitis^[119]. Relapse was defined by a pouch disease activity index (PDAI)^[120] of 2 points or more and confirmed by endoscopy and histology, and was, strikingly, only identified in 15% of the VSL#3 group as against 100% in the placebo group. In a prophylactic study, 2 of 20 patients (10%) receiving 1 packet of VSL#3 1 year developed pouchitis, versus 40% of placebo treated patients^[121].

Somewhat different conclusions were reached in two recently published studies^[122,123]. In a group of 36 patients with recurrent or refractory pouchitis who had required antibiotics at least twice in the past year, patients were randomly assigned to VSL#3 or placebo after achieving remission, once daily for a year. More patients remained in remission in the probiotic group (85% *vs* 6%)^[122].

In an observational study involving 31 patients treated with VSL#3 after achieving remission with Ciprofloxacin, only a minority of patients remained on probiotic therapy and in sustained remission, having stopped them due to recurrence of disease or adverse effects^[123]. In summary then, the benefit of probiotics in Crohn's disease remains unproven. The benefit of probiotics in ulcerative colitis remains unproven. *E. coli* Nissle 1917 appears promising and may be of value in patients intolerant of or resistant to 5-ASA preparations. Limited data from small controlled studies would suggest that VSL#3 is a reasonable therapy in the primary and secondary prevention of pouchitis.

Probiotics in critical illness

Some advocates propose that probiotics have an important emerging role in managing critical illnesses originating in the gastrointestinal tract. In a recent study from Hungary patients with severe acute pancreatitis were randomised upon arrival to hospital to receive one week of treatment with a twice daily administration of a freeze dried preparation containing 10⁹ live *L. plantarum* 299 with a substrate of 10 g oat fibre, or a similar preparation containing *Lactobacillus* which had been inactivated^[124]. The study was stopped when the infection rate showed a significant difference in the two groups. This occurred when 45 patients had completed the study, 22 had received treatment with live and 23 with heat killed *L. plantarum* 299. Infected pancreatic necrosis occurred in 1 out of 22 subjects (4.5%) in the treatment group as against 7 of 23 (30%) in the heat killed group. The length of hospital stay was shorter in the live LAB group but it did not reach statistical significance due to small sample size^[124].

Studies on cirrhotic patients have shown a decrease in the incidence of encephalopathy. A Chinese study recently reported 55 patients with minimal hepatic encephalopathy

who were randomised to receive a synbiotic preparation, fermentable fibre alone or placebo. Synbiotic treatment was associated with a reduction in serum ammonia, endotoxaemia, reversal of encephalopathy and improvement in Child-Turcotte-Pugh score in 50% patients^[125]. Following liver transplantation, bacterial and fungal infections may occur in the first month despite extensive antibiotic treatment and selective digestive tract decontamination. A German study showed a reduction in post-transplant infective complications using probiotics^[126]. Another recent study looked at the effects of a synbiotic preparation on gut barrier function and in critically ill patients admitted to the Intensive Care Unit (ICU). Ninety patients admitted to ICU were randomised (45 in each group) to receive either synbiotic preparations (*Lactobacillus acidophilus* La5, *Bifidobacterium lactis* Bb12, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* with oligofructose (as a prebiotic) or placebo. Patients in the synbiotic group had a lower incidence of potentially pathogenic bacteria (43% vs 75%, $P = 0.01$) and multiple organisms (39% vs 75%, $P = 0.01$) but there were no significant differences in both groups in terms of intestinal permeability, septic complications or mortality^[127]. It should be pointed out that the study lasted only one week.

Probiotics and colon cancer

Colon cancer is one of the leading causes of death in the Western world. Dietary intake of red meat is probably associated with a higher risk whereas the intake of fruit, vegetables, fish and calcium are arguably associated with a lower risk. It is interesting that colon cancer risk is also lower in countries such as Netherlands and Finland where a larger quantity of yoghurt is consumed^[128,129]. Could diet exert its effects *via* the microflora? If so, mechanisms involved might include altered metabolic activity of the intestinal microflora, binding and degradation of potential carcinogens, production of antitumorigenic or mutagenic compounds and enhancement in host immune response. In animals where colon cancer was induced by chemical carcinogens, administration of lactic acid bacteria resulted in a suppression of DNA damage, tumour formation and growth^[130-133].

One bio-epidemiological study showed a higher risk of colon cancer in the simple presence of *Bacteroides* species, but lower risk with *Lactobacillus acidophilus*, *Lactobacillus* S06 and *Eubacterium aerofaciens* identifiable in faecal flora^[134]. This raises the intriguing hypothesis that similarly shifting the resident bacterial populations may be accompanied by parallel reductions in neoplastic risk. However, biodiversity in the colon may merely represent an epiphenomenological consequence of the dietary and environmental risk factors listed above.

Probiotics and digestion

Probiotics may have a promising role in certain aspects of human digestion as illustrated by some interesting studies. Lactose digestion has been shown to improve in lactose malabsorbers who consume live yoghurt rather than milk^[135,136]. The beneficial effects of yoghurt in lactose malabsorbers may result from improved digestion of lactose in the colon from stimulation of colonic bacterial activity by lactic acid bacteria^[137].

Probiotics in irritable bowel syndrome

Irritable bowel syndrome (IBS) is a collection of functional gastrointestinal symptoms such as abdominal pain, defaecatory frequency and or constipation. This area is inevitably contentious, since it remains unclear how much intrinsic intestinal pathology exists in IBS. However, changes in gut sensitivity and defaecatory function are clearly present. Alterations in the composition of intestinal flora have been reported but not proven including a decrease in faecal *Lactobacilli*, *E. coli* and *Bifidobacteria*^[41,110,111] and an increase in other faecal anaerobes^[138-140]. Symptomatic improvement was noted in a very small crossover trial of 18 patients with IBS given *L. acidophilus*^[141] and in an uncontrolled study using *E. faecium* PR88^[142]. On the other hand, no improvement was seen in bloating, pain or urgency to defaecate after the consumption of *Lactobacillus* GG for 8 wk^[143,144]. In a study reported by Saggioro *et al*^[145], 50 patients with IBS according to Rome II criteria were randomly assigned to a probiotic preparation (a combination of *Lactobacillus plantarum* LPO 1 and *Bifidobacterium breve* Bro or placebo for 4 wk). Pain and severity scores decreased significantly after 14 d of treatment. In a more recent study, 77 patients were randomly assigned to a malted drink containing *Lactobacillus salivarius* UCC4331 or *Bifidobacterium infantis* 35 624 or a malted drink alone^[146]. Significant improvement in symptoms was noted in the *B. infantis* group. A corresponding normalisation in the ratio of IL-10/IL 12 was also noted suggesting that the probiotic may help reduce a proinflammatory state associated with IBS.

However the heterogeneity of the various studies makes it difficult to draw conclusions on the effect of probiotics in IBS, and the field is bedevilled by the fact that all therapeutic interventions in IBS produce a 30%-50% placebo response.

Probiotics and *Helicobacter pylori*

H. pylori infection is associated with gastritis, gastro duodenal ulcers and gastric malignancies. The majority of *H. Pylori* hosts become hypochlorhydric with time. Clinical studies and experimental models have shown that the secreted products of *Lactobacillus acidophilus* can suppress *H. Pylori* growth *in vitro* and *in vivo* *L. johnsonii*^[147] and LG21^[148] are effective in suppressing the growth of *H. pylori* and reducing gastric inflammation. Placebo controlled studies have demonstrated a reduction in side effects of standard triple therapy if probiotics were administered concurrently^[149-151]. Daily intake of inactivated *L. acidophilus* was shown to improve the efficacy of eradication treatment^[152]. Only one study^[153] showed that supplementation with fermented milk, containing live special probiotic *L. casei* DN-114001, confers an enhanced therapeutic benefit on *H. pylori* eradication in children with gastritis on triple therapy. The theory that probiotic therapy enhances the disappearance of *H. pylori* does not gain any strength from the available literature. Further clinical studies would be needed to evaluate the effects of long term ingestion of probiotics in preventing *Helicobacter*-associated diseases, but are unlikely to supplant *H. pylori* eradication which is rapidly and

highly effective.

In conclusion probiotics are live microbial food supplements or components of bacteria which may have beneficial effects on gastrointestinal health. Innate bacterial floras clearly play an important role in reinforcement of the physical gut barrier, affecting paracellular permeability, mucosal trophic action and microbiological interactions with mucosal lining of the gut. The key and unanswered question is whether the deliberate manipulation of the bacterial complement in the gut can confer clinical benefit. Probiotics do now appear to have a potential role in the prevention and treatment of various gastrointestinal illnesses, but it is likely that benefits achieved are specific to the bacterial species used and to the underlying disease context. Much further work is required from bench to bedside before we can realise the potential of these new interventions.

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REVIEW

H. pylori and gastric cancer: Shifting the global burden

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Abstract

Infection with *H. pylori* leads to a persistent chronic inflammation of the gastric mucosa, thereby increasing the risk of distal gastric adenocarcinoma. Numerous studies have determined a clear correlation between *H. pylori* infection and the risk of gastric cancer; however, general eradication is not recommended as cancer prophylaxis and time points for treatment remain controversial in different areas of the world. Prevalence rates in Western countries are decreasing, especially in younger people (< 10%); and a decline in distal gastric adenocarcinoma has been observed. Risk groups in Western countries still show considerably higher risk of developing cancer, especially in patients infected with *cagA*⁺ strains and in persons harboring genetic polymorphism of the *IL-1B* promoter (-511T/T) and the corresponding *IL-1* receptor antagonist (*IL-1RN**2). Thus, general eradication of all infected persons in Western countries not recommended and is limited to risk groups in order to achieve a risk reduction. In contrast, infection rates and cancer prevalence are still high in East Asian countries. A prevention strategy to treat infected persons may avoid the development of gastric cancer to a large extent and with enormous clinical importance. However, studies in China and Japan indicate that prevention of gastric cancer is effective only in those patients that do not display severe histological changes such as atrophy and intestinal metaplasia. Thus, prophylactic strategies to prevent gastric cancer in high risk populations such as China should therefore especially aim at individuals now at younger age when the histological alterations caused by the bacterial infection was still reversible. In countries with a low prevalence of gastric cancer, risk groups carrying *cagA*⁺ strains and *IL-1* genetic polymorphisms should be identified and treated.

INTRODUCTION

Since the discovery of *H. pylori* in 1983, intensive research has led to the conclusion that infection with this bacterium is the major cause for the development of distal gastric cancer. Infection with the bacterium leads to a chronic inflammation of the gastric epithelium, associated with multifocal gastric atrophy, dysplasia, and malignant transformation in some of the infected patients^[1-6]. Controversy remains why only a minority of infected patients develops distal adenocarcinoma, and how geographic differences between Western and Asian countries may contribute to these differences. *H. pylori* infection rates average at about 30% in Western populations; in this part of the world, approximately 0.1%-1% of the patients with *H. pylori* induced gastritis will develop distal gastric cancer^[7,8]. Infection rates in Asian countries are higher and range at 60%-88%; distal gastric adenocarcinoma is even more frequent in these countries. Overall, gastric cancer was responsible for almost 650 000 deaths worldwide in the year 2000^[9]. Death from gastric cancer is second only to lung cancer in men and thus contributes to approximately 10% of all cancer deaths annually (see: www.who.org). Since the diagnosis of early gastric cancer is difficult and in most cases diagnosis is made at a more progressed stage, treatment becomes cost-intensive. Therefore, there is a considerable interest to understand the underlining mechanisms and to find strategies to eradicate *H. pylori* infection which could prevent gastric carcinogenesis. Such statistical calculations have already been anticipated 10 years ago in high risk populations of Japanese immigrants in Hawaii; currently, however, no guidelines exist when and whom to treat^[10,11].

EPIDEMIOLOGICAL FEATURES IN EAST AND WEST

H. pylori infection and gastric cancer in Western countries

In Germany in the year 2000, 21 000 patients suffered from gastric cancer with an equal distribution among males and females^[12]. In the US (<http://seer.cancer.gov>),

a similar occurrence has been reported with more than 45 000 deaths per year. In countries such as the USA or Germany, the prevalence of *H. pylori* is decreasing, so that meanwhile less than 10% of the children are infected with this bacterium, underlining that a prophylactic eradication or vaccination in childhood is of little interest in these countries^[8,13-15]. Nevertheless, infection rates are high in elderly populations above the age of 50, and this group is particularly suffering from gastric cancer. A reduction of cancer risk may be achieved when patients are cured from the infection. It may thus be important to identify those patients who are at increased risk.

Gastric cancer in Japan and East Asia

Gastric cancer is the second highest cause of cancer-related deaths in Japan, and the death rate due to gastric adenocarcinoma has actually marginally increased to approximately 50 000 deaths per year in this country^[13,14,16]. In China, a similar prevalence of gastric cancer has been reported. *H. pylori* infection rates in these countries are high and range from 60% in the younger population (10-40 years old) to 80%-90% in elderly patients above the age of 50. In Japan, mortality from distal gastric adenocarcinoma is among the highest scores of the world and could be attributed to the very high rates of *H. pylori* infection in this country and the fact that the most common strain of *H. pylori* found in Japan is extremely virulent. A prophylactic eradication in people at younger age could save millions of lives as well as enormous costs resulting from ulcer disease, gastrointestinal bleeding and gastric cancer treatment. Therefore, a successful eradication of this pathogen is of crucial socio-economical importance especially in countries such as Japan or China, particularly especially in persons who are at age < 50 years.

The "Asian paradox"

Although there is a considerable high rate of *H. pylori* infection in Asian countries such as China, Japan, Thailand and Indonesia, there is a remarkable difference regarding the outcome of gastric cancer within these countries. This observation has also been termed as the "Asian paradox": Although there is a high infection rate in Thailand and Indonesia, there is only little risk of developing gastric cancer in these countries^[14,17]. As discussed below, bacterial virulence factors as well as the individual host immune response may be different among those countries and account for the differential development of the infection outcome. Questions remain whether to treat all infected patients, subgroups of patients, or wait until serious histological alterations have occurred in the various countries mentioned above. The outcome of infection, however, is difficult to predict. Recent studies have elucidated some of the mechanisms of chronic inflammation and thus enable a more precise prediction of the clinical course of infection in different countries.

Impact of strain types on development of distal gastric adenocarcinoma

In recent studies, a new analytical investigation to overcome a potential underestimation of the association of *H. pylori* with gastric cancer was performed in a

Western population. Applying various more stringent exclusion criteria to minimize a potential bias from this source increased the odds ratio (95% confidence interval) of non-cardia gastric cancer from 3.7 to 18.3 for any *H. pylori* infection, and from 5.7 to 28.4 for *cagA*⁺ *H. pylori* infections in Germany^[18-20]. A similar approach has been performed in a study from Sweden, and the results were remarkably consistent with the German study. The observations made in these studies are further supported by a recent cohort study from Japan, in which 36 out of 1246 *H. pylori* infected subjects, but none of 280 uninfected subjects developed gastric cancer during a mean follow-up of 7.8 years^[21]. These very stringent epidemiological studies strongly support that the *H. pylori* gastric cancer association may have been strongly underestimated the risks in previous studies, and consequently, underline that infection especially with certain *cagA*⁺ *H. pylori* can be considered as a true carcinogenic factor. Determination of *cagA* status may thus help the physician to identify people who are at increased risk for gastric cancer. Meanwhile, tests have become available as a serological test and a specific antibody staining for histological specimens^[16].

IMPORTANCE OF BACTERIAL STRAIN TYPES: MOLECULAR AND IMMUNOLOGICAL MECHANISMS

Bacterial virulence factors, especially genetic diversity in the *cagA* region, have been claimed to account for this diverging development. The *H. pylori* *cag* pathogenicity island (*cagPAI*) is a 35 to 40 kb genetic element that encodes a type IV secretion system. One of the key factors of this system is the CagA protein; *cagA*⁺ strains inject the CagA protein directly into host cells where it undergoes tyrosine phosphorylation by a host-cell kinase, and the phosphoprotein alters the physiology of the affected cells. Huang *et al* conducted a meta-analysis of the relationship between CagA seropositivity and gastric cancer^[22]. Based on 7 studies with 1707 gastric cancer patients and 2124 matched controls the analysis showed that infection with *cagA*⁺ *H. pylori* strains increases the risk for gastric cancer 2.87 fold over the risk associated with *H. pylori* infection alone.

Hatatekayama *et al* recently reported that CagA proteins isolated in East Asia, where gastric cancer is prevalent, have a distinct sequence at the phosphorylation site compared with CagA proteins from Western *H. pylori* strains^[23-25]. This CagA diversity may be one important variable in determining the biological activity of CagA and the clinical outcome of infection. Translocated CagA forms a physical complex with the SRC homology 2 domain (SH2)-containing tyrosine phosphatase (SHP-2) in a phosphorylation-dependent manner, and stimulates phosphatase activity. SHP-2 is known to play an important inductive role in mitogenic signal transduction. Deregulation of SHP-2 by CagA may induce abnormal proliferation of gastric epithelial cells. In addition, the CagA protein is polymorphic. Thus, CagA proteins isolated in northern parts of East Asia, where gastric cancer is prevalent, have a distinct sequence at the phosphorylation site and thus contribute to the differential outcome.

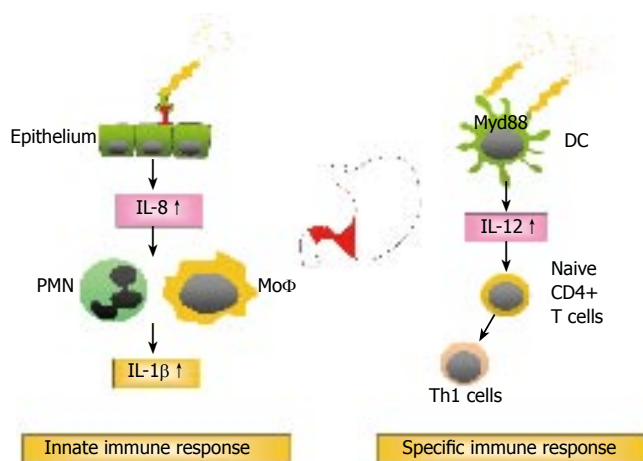


Figure 1 *H pylori*: Immune response and suppression.

Importance of bacterial adherence factors

cagA⁺ *H pylori* strains also are more likely to express the *babA* product, which mediates adherence to Lewis^b antigens on gastric epithelial cells^[26]. European studies have determined that bacteria expressing the adherence factor BabA are detected more frequently in patients with gastric cancer or severe histological changes in the mucosa. Comparing several independent studies in different countries, the relative risk for the development of distal gastric adenocarcinoma was increased up to 20-fold. These results support the hypothesis, that the risk of developing severe gastric pathologies is dramatically increased once the correct determination of the *cagA* status is performed^[27-31].

Current model for the initiation of heightened gastric inflammation

A new concept has now emerged why *cagA*⁺/*babA*⁺ *H pylori* strains induce higher levels of the proinflammatory cytokines IL-1β and IL-8. Adherence of *H pylori* to epithelial cells *via* BabA favors a more tight adhesion to the epithelial cell and thus promotes bacterial colonisation. Injection of CagA into epithelial cells is associated with IL-8 secretion, which in turn, acts as a local chemoattractant for polymorphic mononuclear cells (PMN)^[29]. These PMNs are considered to be of critical importance for the breakdown of the local epithelial barrier and may lead to a further infiltration of the bacteria into the submucosa. Thus, several studies outline the view that bacterial virulence and adherence factors contribute to the development of severe diseases in the stomach. Infection with certain *H pylori* strain types is therefore more dangerous than smoking a pack of cigarettes per day, leading to a 21-fold increased relative risk for the development of lung cancer.

Figure 1: Events during the initial infection process with *H pylori*

Infection with so called type 1 strains harboring *cagA*/*vacAs1* and *babA* is associated with a dramatically increased risk to develop distal gastric adenocarcinoma. Infection with specific strain types thus is the major determinant of the further sequence of events.

IMPORTANCE OF HOST GENETICS FOR THE *H PYLORI* INDUCED INFLAMMATION AND CANCER DEVELOPMENT

Genetic polymorphisms contribute to severe gastric inflammation

In addition to bacterial factors, but less important in terms of relative risk increment, are host factors that seem to influence the inflammatory response and the development of a more severe pathology. *H pylori* induced inflammation is implicated in the development of mucosal damage and is characterised by strong granulocytic and lymphocytic infiltration. The T helper cell response towards *H pylori* is generally considered to be of the Th1 phenotype, leading to a cell mediated immune response^[32-35] (Figure 1). There is increasing evidence that the *H pylori* induced Th1 response contributes to cancer development. Downregulation of the Th1 response in mice by concurrent enteric helminth infection or p53 mutation was shown to protect against the development of atrophy, intestinal metaplasia, and invasive gastric carcinoma^[35-37]. Important cytokines characterising Th1 mediated immune responses are interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), all being upregulated during chronic *H pylori* infection^[38-40]. IL-10, which is also highly expressed in the *H pylori* infected stomach, is one of the most important regulatory cytokines, inhibiting cell mediated immune responses^[38,41,42].

Genes encoding cytokines and related molecules harbour polymorphic regions, which are considered to alter gene transcription and thereby influence inflammatory processes in response to infectious diseases. Polymorphisms in the human *IL-10*, *IL-1B*, *TNF-α*, *IFN-γ*, and *IL-1* receptor antagonist (*IL-1RN*) genes have been reported to influence cytokine expression^[43-47]. In the promoter region of the *IL-1B* gene, *IL-1B-511T*, which is in complete linkage disequilibrium with *IL-1B-31C*, was previously associated with slightly, but not significantly, increased IL-1β secretion from stimulated PBMC. The *IL-1RN* gene has a penta-allelic 86 bp variable number of tandem repeat region (VNTR) in intron 2, of which allele 2 (*IL-1RN*2*) was previously associated with enhanced IL-1β secretion^[46] and the development of gastric cancer^[44]. Presence of certain genetic polymorphisms of the host, in contrast to the bacterial factors, increase the relative risk for distal gastric adenocarcinoma by 1.5-4 fold. In a German population^[48], the homozygous genotype *IL-1RN*2/2* of the *IL-1RN* gene was strongly associated with early-stage gastric cancer ($P < 0.0001$), whereas further associations with the *IL-1* gene cluster were not observed. A Korean group^[49] determined that patients with intestinal-type gastric cancer showed a higher frequency of *IL-1B-31T* homozygotes [odds ratio (OR) = 2.2; 95% confidence interval (CI) = 1.1-4.3] compared with controls. Risk was also significantly increased in these patients for *IL-1B-31T* homozygotes compared with patients with diffuse-type gastric cancer (OR = 3.4; 95% CI = 1.5-7.7). In a Chinese study, it was reported that the relative risks associated with the *IL-1B* variant genotypes were 1.64

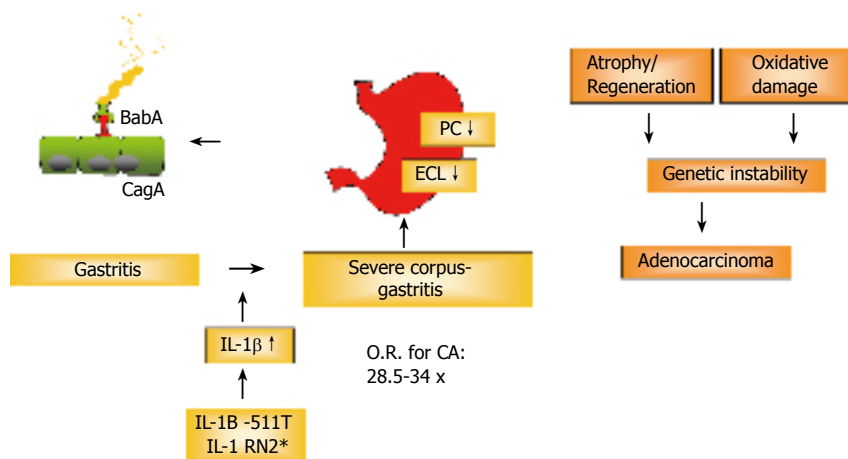


Figure 2 *H. pylori*: Chronic inflammation and carcinogenesis.

(95% CI, 1.01-2.66) for -31TT and 1.52 (95% CI, 0.91-2.54) for -511CC, respectively, compared with their wild-type homozygotes^[50]. The risks were significantly more evident in individuals with *H. pylori* infection (adjusted OR = 2.14; 95% CI, 1.13-4.06 for -31TT; adjusted OR = 2.00; 95% CI, 1.02-3.89 for -511CC), which was consistent with the biological effects of IL-1 β .

Chen *et al.*^[51] identified that the carriage of IL-1RN*2 polymorphism, male gender, old age and *H. pylori* infection independently increased the risk of gastric cancer, with odds ratios of 3.3 (95% CI, 1.4-7.7), 2.1 (95% CI, 1.2-3.8), 5.3 (95% CI, 3.1-9.0) and 2.2 (95% CI, 1.3-3.8), respectively. *H. pylori*-infected individuals who were carriers of IL-1RN*2 showed increased risks for both intestinal and diffuse types of gastric cancer, with odds ratios of 11.0 and 8.7, respectively. Thus, IL-1RN*2 was interpreted to be an independent factor governing the development of gastric cancer in Asian individuals.

In areas of high and low prevalence of gastric cancer in China^[52], the IL-1B -511T/T genotype frequency was significantly higher among patients with gastric cancer (25.0%) than control subjects (12.5%) in a region with low GC prevalence. While *H. pylori* infection alone had only a modest effect on the risk of gastric cancer development (OR = 5.0, 95% CI 1.5-16.3), combined with the IL-1B -511T/T genotype the risk was markedly elevated (OR = 17.1, 95% CI 3.8-76.4). The study underlines the synergy between *H. pylori* infection and the host immune response to induce gastric cancer.

A recent German study confirmed this synergistic effect also in regard to the induction of premalignant changes. In a large group of *H. pylori* infected patients^[29], carriers of the proinflammatory IL-1B -511T/-31C and IL-1RN*2 alleles had an increased risk for the development of atrophic gastritis (AG), intestinal metaplasia (IM), and severe inflammation, ORs of 1.7 (95% CI, 0.8-3.4) to 4.4 (95% CI, 1.5-12.9). The highest prevalence of severe gastric abnormalities was found in patients with both host and bacterial high-risk genotypes (*cagA*⁺/*vacAs1*⁺/IL-1B -511T/IL-1RN*2), with ORs of 24.8 (95% CI, 5.2-117.3) for severe lymphocytic infiltration, 9.5 (95% CI, 2.8-32.1) for severe granulocytic infiltration, 6.0 (95% CI, 2.4-15.5) for IM, and 2.4 (95% CI, 0.93-6.2) for AG. These dramatically elevated values underline the importance

of the infection for gastric cancer development. It may thus be concluded from the above studies, that combined bacterial/host genotyping thus may provide a clinical tool to identify patients at high risk of developing gastric cancer. This current concept of gastric carcinogenesis is illustrated in Figure 2.

Figure 2: Concept of gastric carcinogenesis

There is overwhelming evidence that *H. pylori* infection, especially with *cagA*⁺ strains, leads to a strong granulocytic and lymphocytic infiltration; a subgroup of infected patients will develop gastric cancer, especially in high-risk countries such as Japan, China and East-European countries. Reasons for this progression include genetic polymorphisms, e.g. IL-1B/IL-1RN polymorphisms associated with high levels of IL-1 β . IL-1 β is a strong antisecretory cytokine. These genetic polymorphisms lead to a heightened cytokine release, which in turn decreases acid secretion. Subsequently, the association of hypochlorhydric conditions with the persistent inflammation in the gastric corpus is considered to be a true risk factor (RR: 34.5-fold!) for the development of gastric cancer. Studies should therefore aim not only at preventing gastric cancer, but also at preventing the development of severe corpus gastritis, leading to potentially irreversible atrophy and hypochlorhydria.

PROPHYLAXIS OF GASTRIC CANCER

Prospective intervention studies

Data from a Chinese trial^[53] investigating *H. pylori* eradication in gastric cancer showed that the incidence of gastric adenocarcinoma development was similar in patients undergoing eradication therapy compared with placebo over a 7.5-year period. Interestingly, in a subgroup of *H. pylori*-positive patients without atrophy, intestinal metaplasia or dysplasia (pre-cancerous lesions), eradication therapy significantly decreased the development of gastric adenocarcinoma to a frequency of zero. The data are in accordance with a recent study in Japan^[4]: in this prospective study, no carcinomas were observed in the *H. pylori* negative group. This study supports the view that a prospective eradication study should be performed in young adults in which histological alterations have not

proceeded too far and may be reversible.

Zhou *et al*^[54] investigated histopathological changes in *H pylori* eradicated subjects in China over a period of more than 5 years. The authors report in a group of 552 *H pylori*-positive subjects that, the severity and activity of inflammation in both the antrum and body were markedly reduced after *H pylori* eradication. Within the five years after eradication of *H pylori*, intestinal metaplasia in the antrum regressed or showed no progression, while the proportion of intestinal metaplasia in the *H pylori*-positive group increased significantly. After *H pylori* eradication, the atrophy in both the antrum and body had no significant regression. After eight years of observation, the authors report a significantly higher incidence of gastric cancer in the *H pylori* infected group (5/1530) than in the *H pylori*-negative (1/1230) group in the Yantai area of China, underlining the need for eradication in early stages.

Serum pepsinogen levels as risk factors for developing gastric cancer

A recently published study^[55] reports that subjects seropositive for either *H pylori* or CagA who had low pepsinogen (PG) I levels had the highest OR (9.21 95% CI 4.95-17.13) for noncardia cancer, compared with subjects with neither factor. The results suggest that individuals with both *H pylori* or CagA seropositivity and a low PG I level or low PG I / II ratio are highly susceptible to development of noncardia gastric cancer. Such tests can easily be performed in clinical routine. Watabe *et al*^[56] have confirmed that *H pylori* infection and gastric atrophy are both risk factors for gastric cancer in a study group with a total of 9293 participants. The annual incidence of gastric cancer was 0.04%-0.60%. As expected, the highest relative risk for gastric cancer was found in *H pylori* infected subjects with gastric atrophy and high serum pepsinogen levels [OR = 8.2 (3.2-21.5)].

H pylori eradication and the risk of esophageal cancer

Concerns exist whether eradication of *H pylori* might lead to the development of esophageal cancer. To clarify the role of *H pylori* infection in these tumors with divergent incidence trends, Chow *et al*^[57] analyzed serum IgG antibodies to *H pylori* and to a recombinant fragment of CagA by antigen-specific ELISA among 129 patients newly diagnosed with esophageal/gastric cardia adenocarcinoma, 67 patients with noncardia gastric adenocarcinoma, and 224 population controls. Infection with *cagA*⁺ strains was not significantly related to risk for noncardia gastric cancers (OR = 1.4; CI = 0.7-2.8) but was significantly associated with a reduced risk for esophageal/cardia cancers (OR = 0.4; CI = 0.2-0.8). The study thus contradicts previous reports and may be biased by the selection of patients in a rather small group. While Wu *et al*^[58] found no significant association, Ye *et al*^[59] determined the opposite: *H pylori* infection may protect from esophageal cancer (OR = 0.3). Thus, at this point, several works indicate that CagA has a protective effect on the development of esophageal cancer, however, no general conclusions can be made.

However, this association does not justify a general refusal of *H pylori* eradication because of two reasons. First, esophageal adenocarcinoma is far less common

than gastric cancer. Second, the risk of developing adenocarcinoma of the esophagus is lowered by a factor 2-2.5 in the presence of *cagA*-positive *H pylori* infection whereas the risk for distal gastric cancer associated with *cagA*-positive strains is much higher (5-28x).

CONCLUSIONS

H pylori is a clear cut carcinogen. Infection with certain strain types in the presence of genetic polymorphisms leading to a heightened inflammatory response is associated with a dramatically increased relative risk to develop gastric cancer. Such developments can be foreseen by evaluating gastric biopsies, but also by determining serum pepsinogen levels as a marker of gastric atrophy. Endoscopy of the upper gastrointestinal tract (GI) should be performed before eradication to determine the status of gastric inflammation. Eradication of *H pylori* as prophylaxis of gastric adenocarcinoma is effective in early stages in which no severe histological changes have occurred. In countries with a high prevalence of infection and high cancer risks such as Japan or China, a general prophylactic eradication strategy seems to be beneficial especially in younger patients (< 50 years); nevertheless, the outcome of such long term prospective prophylactic studies needs to be evaluated. In Western countries with low prevalence rates and low cancer rates, a test-and-treat strategy is not cost-effective to prevent cancer; however, identification and treatment of risk groups seems rationale. Risk groups can be identified by evaluation of family history, presence of histological alterations in the gastric corpus, or by determining infection with *cagA*⁺ strains as well as determining the status of genetic polymorphisms in the IL-1RN*2 gene.

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H. pylori status and angiogenesis factors in human gastric carcinoma

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Conversely, neither VEGF-R1 expression nor MVD was related to p53 expression. However, *H. pylori* was not related to any angiogenic markers except for the plasma VEGF level ($P = 0.026$).

CONCLUSION: *H. pylori* antigen is related to higher plasma VEGF levels, but not to angiogenic characteristics. It can be hypothesized that the toxic effects of *H. pylori* on angiogenesis occurs in early preclinical disease phase or in long-lasting aggressive infections, but only when high *H. pylori* IgG levels are persistent.

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Key words: *H. pylori*; Gastric carcinoma; Angiogenesis

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Abstract

AIM: To investigate *H. pylori* expression in gastric cancer patients in relation to primary tumor angiogenic markers, such as microvessel density (MVD), thymidine phosphorylase (TP), vascular endothelial growth factor receptor-1 (VEGF-R1), p53 and circulating VEGF levels.

METHODS: Angiogenic markers were analyzed immunohistochemically in 56 primary gastric cancers. *H. pylori* cytotoxin (*vacA*) and the cytotoxin-associated gene (*cagA*) amplification were evaluated using PCR assay. Serum *H. pylori* IgG antibodies and serum/plasma circulating VEGF levels were detected in 39 and 38 patients by ELISA, respectively.

RESULTS: A total of 69% of patients were positive for circulating IgG antibodies against *H. pylori*. *cagA*-positive *H. pylori* strains were found in 41% of gastric patients. *vacA* was found in 50% of patients; s1 strains were more highly expressed among *vacA*-positive patients. The presence of the s1 strain was significantly associated with *cagA* ($P = 0.0001$). MVD was significantly correlated with both tumor VEGF expression ($r = 0.361$, $P = 0.009$) and serum VEGF levels ($r = -0.347$, $P = 0.041$).

INTRODUCTION

H. pylori infection is a well-known risk factor for the development of pre-neoplastic and neoplastic gastric mucosal alterations^[1,2]. An increase in proliferative activity of gastric epithelial cells without a corresponding increase in apoptosis has been implicated in *H. pylori* gastric carcinogenesis^[3,4]. In addition, specific virulence determinants of *H. pylori* strains can influence the outcome of the infection. Urease, vacuolating cytotoxin *vacA*, and the pathogenicity island (*cag* PAI) gene products are the main virulence factors of this organism involved in the development of gastric carcinoma. Thus, individuals infected with strains that express these virulence factors are prone to develop severe local inflammation which may induce the development of peptic ulcers and gastric cancers. Also, *H. pylori* activity may be associated with virulence; in fact, urease activity may be an important colonization factor and exert a direct toxic effect upon intercellular junctions, resulting in alteration of gastric mucosal permeability^[5]. The subsequent passage toward cancer is probably prompted by other factors, such as the onset of infection or other agents independent of *H. pylori*.

Several studies have suggested that angiogenesis might also contribute to gastric tumorigenesis^[6-8]. Angiogenesis is a complex multistep cascade modulated by positive soluble factors, such as the vascular endothelial growth factor (VEGF). The tumor neo-angiogenesis has been demonstrated in almost all solid tumors using various morphological techniques. The current method of angiogenesis quantification is the evaluation of CD34 antigen expression, a cell surface glycoprotein also present in the vascular endothelium permitting the study of intratumor endothelial cells^[9]. The cellular receptor for VEGF, VEGF-R1 or Flt-1, is highly expressed in gastric carcinoma cells, suggesting that this pathway could influence tumor growth and metastasis through paracrine and autocrine mechanisms^[10]. An additional tissue factor is thymidine phosphorylase (TP), an enzyme involved in pyrimidine nucleoside metabolism, which is identical to the platelet-derived endothelial cell growth factor and is endowed with angiogenic activities in various solid tumors^[11]. Furthermore, the *p53* oncosuppressor gene has been reported to be involved in inhibition of tumor vascularization by fostering unopposed angiopoietin-2 activity^[12].

Recent publications have suggested that *H pylori* infection may regulate the angiogenesis and invasion of gastric carcinoma. In fact, *H pylori* influences *in vitro* angiogenesis-related gene expression; in particular, it has been demonstrated to up-regulate VEGF expression in gastric epithelial cells, an effect which appears to be related to *vacA*-expression^[13,14]. Moreover, *H pylori* has been shown to up-regulate the expression of epidermal growth factor (EGF)-related growth factors and COX-2 in *in vitro* human gastric epithelial cells as well as in human gastric mucosa *in vivo*^[15,16]. Lastly, its relationship with *p53*, which has been described as an angiogenesis-related factor, has been documented^[17-20]. In spite of these evidences originating from *in vitro* studies, suggesting a relationship between pathophysiological roles for *H pylori* in the induction of tumor neo-angiogenesis, to our best of knowledge, no data are available in literature in patient series. Our hypothesis was that *H pylori*-related gastric cancer could involve different neo-angiogenic characteristics with respect to tumors without bacterial infection.

To verify the association between *H pylori* infection and different angiogenesis-related characteristics, 56 gastric cancer patients were studied for microvessel density (MVD), thymidine phosphorylase (TP), vascular endothelial growth factor-receptor (VEGF-R1) and *p53* expressions in addition to circulating serum and plasma VEGF levels. *H pylori* was investigated at the molecular and at circulating blood levels.

MATERIALS AND METHODS

Patients

Fifty-six patients (37 men and 19 women; median age 64 years, range 42-83 years) with T₁₋₄ N₀₋₁ M₀₋₁ gastric carcinoma were enrolled in this study. All patients had primary surgery for gastric cancer at National Cancer Institute of Bari. Primary tumor tissues were utilized for the immunohistochemical analysis of MVD, *p53*,

Table 1 Clinicopathological features and distribution of *cagA*, *vacA* and IgG anti-*H pylori* in a series of 56 gastric cancer patients

Clinicopathological features	n
Sex	
Male	37
Female	19
Tumour category	
pT _{1-2/3}	28
pT ₄	28
Location	
Antrum	23
Other	33
IgG anti <i>H pylori</i> (ELISA)	39
IgG - (≤ 7 KU/L)	12
IgG + (> 7 KU/L)	27
<i>cagA</i> (PCR)	56
<i>cagA</i> -	32
<i>cagA</i> +	23
NE	1
<i>vacA</i> (PCR)	56
NEG	28
s1m1	10
s2m2	5
s1m2	9
s1m1/s1m2 ^a	1
NE	3

NEG: Negative; NE: Not evaluable; ^a Multiple genome.

VEGF-R1 and TP expressions.

Formalin-fixed and paraffin-embedded specimen of the primary tumor was selected by the pathologist for each patient on the basis of the quality of morphological preservation and neoplastic cellularity. In accordance with standardized sampling protocols, the sample was comprehensive both at the deeper portions of tumor, as well as the edges of the lesions. Five-micrometer thick sections were cut for immunohistochemical assay and for determination of *H pylori* status by means of polymerase chain reaction (PCR). A section contiguous to those selected for immunohistochemistry and DNA extraction was always stained with haematoxylin and eosin and confirmed by the pathologist as rich in neoplastic cellularity. Enzyme-linked immuno-sorbent assay (ELISA) for IgG antibodies against *H pylori* was performed on blood samples from 39 patients. Circulating VEGF levels were detected by ELISA in serum and plasma of 38 patients. The patients characteristics are shown in Table 1.

DNA extraction and PCR analysis

DNA extraction from paraffin-embedded specimens was performed using the method described by Lin *et al*^[21]. Briefly, samples were incubated with a lysis buffer and proteinase K for 3 h at 55°C. Total DNA was extracted with phenol/chloroform, precipitated with acidic ethanol, and dissolved in sterile water.

Amplification of *cagA* and *vacA*

The extracted DNA was subjected to PCR for detection of *H pylori* genes, *cagA* and *vacA*. The *cagA* gene was amplified using the primers described elsewhere^[22,23]. The *vacA* gene

was amplified using primers described by Atherton *et al.*^[24] which evaluate the mid region (*m*) and the region encoding for the signal peptide (*s*) of the gene. Four different PCR products were obtained: *s1* or *s2* from the *s* region, and *m1* and *m2* from the *m* region. PCR products were analyzed by electrophoresis on 20 g/L agarose gel. Positive and negative controls were examined with each batch of PCR.

Detection of anti-*H. pylori* IgG

An enzyme-linked immuno-sorbent assay was used to detect *H. pylori*-specific IgG serum antibodies (Anti-*H. pylori* EIA Quant- COBAS2- Roche Diagnostics). The anti-*H. pylori* IgG EIA is a second-generation two-step EIA for the detection of IgG antibodies to *H. pylori* in human serum, based on a set of fast protein liquid chromatography-purified cell surface antigens, including the native urease enzyme^[25]. According to the manufacturer, patients were considered positive for IgG against *H. pylori* when IgG value was higher than 7 U/mL.

Immunohistochemistry

Serial sections of paraffin-embedded gastric tissue were deparaffinized and rehydrated. For antigen retrieval, the sections were microwaved at 500 W for 10 min in citrate buffer (pH 6) and endogenous peroxidase activity was blocked with 30 mL/L hydrogen peroxide solution. Adjacent slides were incubated with different monoclonal antibodies. The bound antibody was visualized using a biotinylated secondary antibody, avidin-biotin peroxidase complex, and 3-amino-9-ethylcarbazole (Ultra Vision Detection System anti-Polyvalent, HRP/DAB, Lab Vision Corporation). For negative control sections, primary antibody was replaced with phosphate-buffered saline and processed in the same manner. Gastric carcinomas known to express high levels of CD34, p53, TP and VEGF-R1 proteins were used as positive controls.

Anti-CD34 (QB-END/10, Novocastra Laboratories Ltd) was diluted at 1:50 for 1 h at room temperature as a pan-endothelial marker for MVD analysis. The modified Weidner's method was utilized for the evaluation of MVD according to CD34 endothelial cell immunostaining^[26]. For the microvessel counting, positive stainings for MVD, in five most highly vascularized areas ('hot spots') in each slide, were counted in 400 × fields with an image analysis system (Quantimet 500 Leica; 0.19 mm²/field) and MVD was expressed as the average of the microvessel count in these areas^[27]. Any EC or endothelial cluster positive for CD34 (brown yellow staining) was considered to be a single countable microvessel. Sclerotic areas, both hypocellular and necrotic, within the tumor were not considered for vessel evaluation.

Anti-p53 monoclonal antibodies (PAb 1801, Neo-Markers), grown against human p53 and recognizing wild-type and mutant forms of the p53 protein, were diluted at 1:150 for 1 h at room temperature. Tumor cells expressing p53 immunoreactivity were quantified by evaluating a total of 1000 neoplastic cells in random fields from representative areas. Exclusive nuclear staining was scored as positive. The immunoreactive cells were expressed as percentages^[28]. Anti-TP monoclonal antibodies (P-GF.44C Neo-Markers), recognizing full

length human TP protein, were diluted at 1:100 for 1 h at room temperature. TP positivity was determined at 400 × fields with the image analysis system and was evaluated on the basis of percentage of stained epithelial tumor cells. Tumor cells with moderate or strong staining intensity were counted. TP expression in macrophages was considered an internal positive control. The polyclonal antibody anti-VEGF-R1 (Flt-1 polyclonal rabbit antibody, Santa Cruz Biotechnology Inc.), recognizing the carboxyl terminus of the receptor for VEGF, VEGF-R1 or the Flt-1 protein of human origin, was used at a dilution of 1:100 for 1 h at room temperature. VEGF-R1 positivity was scored as cytoplasmic immunostaining using an image analysis system (Quantimet 500 Leica). Immunoreactivity was expressed as the percentage ratio between the area of immuno-positive tumor cells and the entire area of invasive neoplastic tissue.

The laboratory, where the immunohistochemical analyses were performed, participated to Quality Control programs managed by INQAT^[29].

Circulating VEGF detection

Blood samples were collected before surgery. Venous blood was dispensed into a serum separator tube (Becton Dickinson Vacutainer Systems) for serum obtainment, and into sodium citrate, theophylline, adenosine, dipyridamole (CTAD) tubes for plasma (Becton Dickinson Hemogard Vacutainer Systems).

Circulating VEGF levels were examined in plasma and serum using the Quantikine Human VEGF-enzyme-linked immuno-sorbent assay (ELISA, R&D System Inc.) which recognizes VEGF165. According to the manufacturer, the minimum detectable dose of VEGF is typically less than 9.0 ng/L. Values below 9.0 ng/L were equal to zero. VEGF levels in plasma and serum were analyzed, as we previously demonstrated that the two determinations provide alternative and additional information on circulating VEGF, also in relation to the role played by the activation and quantity of platelets in VEGF release^[30].

Statistical analysis

The associations between MVD, TP, VEGF-R1 and p53 expression, markers of *H. pylori* status and histological diagnosis were evaluated using the Chi-square test. A correlation analysis among the aforementioned biomarkers, considered as continuous variables, was performed by Pearson's correlation coefficient (*r*). In the statistical analysis, *vacA* genotypes were classified into two subgroups: "cytotoxic strains" which included s1m1, s1m2 and s1m1/s1m2 strains and "others" which included negative and s2m2. Patients with s1m1/s1m2 strains were infected by multiple genotypes. Backwards stepwise logistic regression analysis was used to estimate the independent association of any biological markers with *H. pylori* characteristics. Statistical analysis was carried out using the software SPSS for windows, release 9.0.

RESULTS

H. pylori status

Cytotoxin-associated gene (*cagA*)-positive *H. pylori* strains

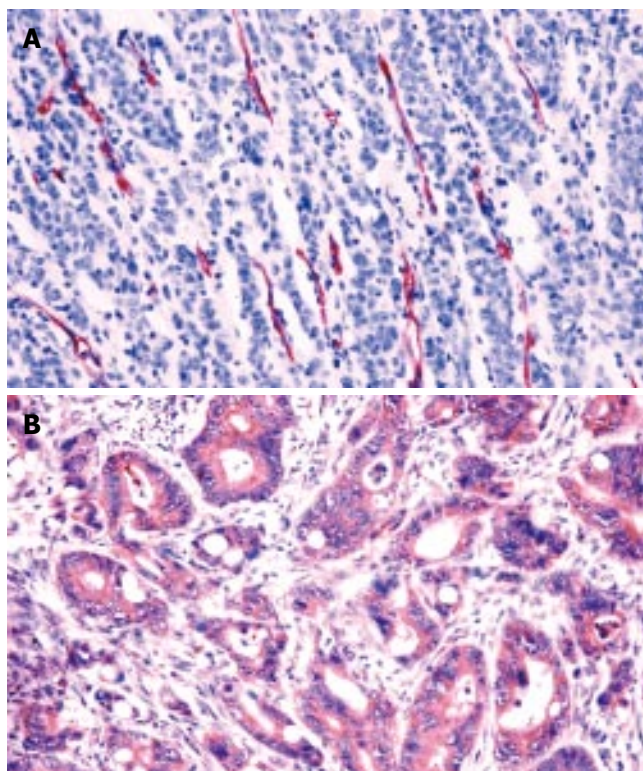


Figure 1 Immunohistochemical assay for detection of microvessel density (A) and VEGF-R1 protein expression (B) in gastric cancer. VEGF-R1 is stained in the cytoplasm of cancer cells.

were found in 41% of gastric patients. *vacA* was found in 50% of patients; s1 strains were more highly expressed among *vacA*-positive patients. Moreover, a single patient was found to be infected with different *H pylori* strains (multiple genomes). The results are summarized in Table 1. The presence of the s1 strain was significantly associated with *cagA* ($P = 0.0001$). Only one patient infected with the s2 strain showed *cagA*-positivity, while 26 (81%) patients were negative for both *cagA* and *vacA*. A total of 69% of patients were positive for circulating IgG antibodies against *H pylori*, with a mean and median value of 77 U/mL (range 1-476 U/mL) and 15 U/mL, respectively.

Immunohistochemical analysis

TP immunoreactivity was observed in normal epithelial cells, malignant epithelial cells, macrophages and endothelial cells. The usual pattern of positive staining was both cytoplasmic and nuclear. A mean of 5% (range 0%-80%) of cells showed TP positivity.

p53 expression was generally confined to neoplastic tissues, while the normal mucosa was rarely stained. A mean of 3% (range 0%-50%) of cells showed p53 positivity.

CD34 immunostaining was detected in the endothelial cells, especially in the area surrounding the tumor (Figure 1A). In the 'hot spot' tumor area, a mean of 39 vessels (range 0-100 vessels) was found with only 2% of cases not demonstrating any microvessels. VEGF-R1 immunostaining was mainly localized at the membrane and cytoplasm of epithelial and endothelial cells (Figure 1B). By counting only the epithelial component, a mean of 24% (range 0%-100%) of VEGF-R1-immunostained cells was found.

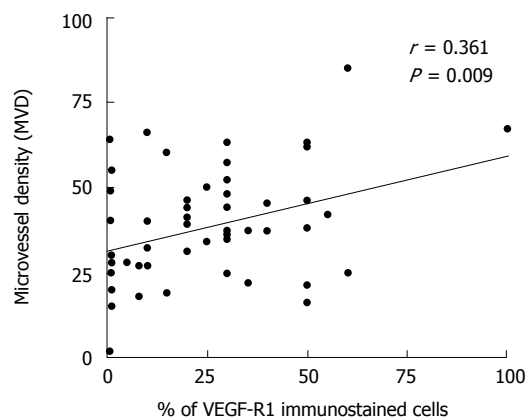


Figure 2 Correlation between percentage of VEGF-R1-immunoreactive cells and microvessel density (MVD) within each tumor.

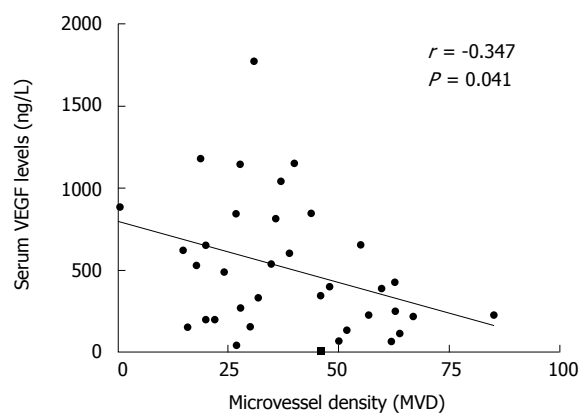


Figure 3 Correlation between serum VEGF levels and microvessel density (MVD) within each tumor.

About 80% of tumors demonstrated VEGF-R1 expression.

Regarding the relationship among the different angiogenic characteristics, only MVD was significantly correlated with both tumor VEGF expression ($r = 0.361$, $P = 0.009$; Figure 2) and serum VEGF levels ($r = -0.347$, $P = 0.041$; Figure 3). Conversely, neither VEGF-R1 expression nor MVD was related to p53 expression.

Table 2 shows the association between the markers of *H pylori* status and angiogenic factors. There was no significant association between markers of *H pylori* status, *cagA*, *vacA* and angiogenic biomarker expression. When the correlation between angiogenesis related-markers and IgG status was analysed a significant correlation between IgG status and plasma VEGF levels was observed ($P = 0.026$). The lack of association between *H pylori* characteristics and biomarkers was also confirmed with multivariate logistic regression analysis.

Angiogenic marker expression and markers of *H pylori* status were analyzed with respect to clinico-pathological features. Plasma VEGF levels and tumor TP expression were both significantly associated with tumor size ($P = 0.030$ and $P = 0.035$, respectively; Table 3). Regarding plasma VEGF levels in particular, T₄ tumors showed a significantly smaller percentage of low IgG cases as compared with T₁₋₃ tumors (31% vs 74%; $P < 0.03$).

Table 2 *cag A*, *vac A*, IgG anti-*H pylori* and angiogenic factors in gastric cancer patients

	IgG anti <i>H pylori</i>		<i>cag A</i>		<i>vac A</i>	
	<i>n</i> (%) With IgG- (≤ 7 U/mL)	<i>n</i> (%) With IgG+ (> 7 U/mL)	<i>n</i> (%) Negative for <i>cagA</i>	<i>n</i> (%) Positive for <i>cagA</i>	<i>n</i> (%) Cytotoxic strains (s1; m1/2)	<i>n</i> (%) Others (absent; s2m2)
TP expression ¹						
0 ²	9 (31)	20 (69)	24 (57)	18 (43)	24 (60)	16 (40)
> 0	2 (25)	6 (75)	8 (80)	2 (10)	8 (80)	2 (20)
p53 expression ¹						
0 ²	8 (25)	24 (75)	27 (60)	18 (40)	28 (65)	15 (35)
> 0	3 (60)	2 (40)	5 (63)	3 (37)	4 (50)	4 (50)
MVD (CD34) ¹						
≤ 37 ²	7 (32)	15 (68)	16 (59)	11 (41)	15 (58)	11 (42)
> 37	3 (21)	11 (79)	16 (64)	9 (36)	17 (71)	7 (29)
VEGF-R1 expression ¹						
≤ 20 ²	6 (29)	15 (71)	16 (59)	11 (41)	14 (54)	12 (46)
> 20	5 (31)	11 (69)	15 (58)	11 (42)	17 (68)	8 (32)
pVEGF levels						
≤ 26 ²	9 (50) ^a	9 (50) ^a	10 (53)	9 (47)	11 (61)	7 (39)
> 26	2 (13) ^a	13 (87) ^a	9 (60)	6 (40)	9 (60)	6 (40)
sVEGF levels						
≤ 432 ²	5 (29)	12 (71)	11 (61)	7 (49)	12 (71)	5 (29)
> 432	6 (32)	13 (68)	9 (47)	10 (53)	9 (47)	10 (53)

sVEGF: Serum VEGF; pVEGF: Plasma VEGF; ¹ % of immunostained cells; ² Cut-off median value of the series; ^a*P* = 0.026.

Table 3 Association between angiogenic characteristics, markers of *H pylori* status and clinicopathological features

Biomarkers	Tumor stage			M status		
	T ₁₋₂₋₃ (<i>n</i> = 28)	T ₄ (<i>n</i> = 28)	<i>P</i>	M ₀ (<i>n</i> = 38)	M ₁ (<i>n</i> = 18)	<i>P</i>
<i>cagA</i> negative (<i>n</i> = 32)	54	63	NS	58	59	NS
<i>vacA</i> no cytotoxic strains (<i>n</i> = 33)	62	63	NS	58	71	NS
TP negative (<i>n</i> = 42)	93	65	0.035	83	71	NS
p53 negative (<i>n</i> = 46)	78	93	NS	78	100	0.038
low MVD values (<i>n</i> = 27)	56	46	NS	54	44	NS
Low VEGF-R1 expression (<i>n</i> = 27)	56	44	NS	51	47	NS
High IgG levels (<i>n</i> = 27)	65	74	NS	77	54	NS
Low pVEGF levels (<i>n</i> = 19)	74	31	0.03	52	58	NS
Low sVEGF levels (<i>n</i> = 19)	35	67	NS	50	50	NS

NS: Non-significant.

Lastly, p53 expression was significantly associated with metastatic status (*P* = 0.038), as 100% of patients with metastatic disease did not express p53. No association was found between cytohistological tumor grading or *H pylori* infection site and angiogenesis-related markers and *H pylori* characteristics.

DISCUSSION

The role of *H pylori* in gastric cancerogenesis has been extensively investigated; conversely, information is lacking regarding the biological impact of *H pylori* on the progression of gastric cancer. Several factors emphasize the importance that various *H pylori* components can have their roles on the development of pre-neoplastic and neo-

plastic alterations of the gastric mucosa. Specific virulence factors produced by the bacterium, such as the vacuolating cytotoxin (*vacA*) or the cytotoxin-associated protein (*cagA*), contribute to gastroduodenal mucosal injury and impair the healing process of the damaged mucosa^[31,32]. In addition, the host response to the infection and the presence of environmental factors are thought to be involved in the pathogenesis of *H pylori*-related gastroduodenal disease^[33,34]. The vacuolating toxin (*vacA*) is believed to be a major determinant of *H pylori*-associated gastric disease^[35,36]. The vacuolating cytotoxin gene A (*vacA*), which encodes the *vacA* protein, is present in all *H pylori* strains, but its encoded products are associated both with and without *in vitro* vacuolating activity^[37]. It has been suggested that the *vacA* s1a genotype is closely associated with

high cytotoxin production, while the *vacA* s2 allele can demonstrate a negative *in vitro* association with cytotoxin activity. The presence of these virulence factors can be used to identify patients at risk to develop gastric cancer; in fact, patients with neoplastic transformation of the gastric mucosa are more likely to be infected by the *cagA*+ strain^[19,38,39]. However, conflicting results regarding the association between these virulence factors and clinical outcome of gastric cancer are found in the literature^[36,40-43].

p53 mutations and the genotypic characterization of *H. pylori* have also been thoroughly studied to identify possible links between *H. pylori* infection and p53 alterations without reaching definitive conclusions. Alterations of the *p53* gene and/or its abnormal protein accumulation have both been described during the later stage of gastric carcinogenesis^[44,45] and in precancerous gastric lesions^[46]. As *cagA*+ *H. pylori* strains induce particularly severe inflammation in the gastric mucosa^[41,47], it has been hypothesized that gastric tumors from subjects infected with *cagA*+ *H. pylori* might have a higher prevalence of p53 mutation than tumors from non-infected subjects.

H. pylori infection may also regulate the angiogenesis and invasion of gastric carcinomas^[13,14], but whether *H. pylori* exerts its effects to induce neovascularization early in the development of gastric pre-neoplastic lesions or late in clinical phases of the disease is still unknown. However, it is clear that *H. pylori* infection can increase the expression of the platelet-derived endothelial cell growth factor by infiltrating interstitial cells in pre-malignant lesions, such as intestinal metaplasia, thereby assisting in creating a favourable environment for tumor development^[48]. Furthermore, it has been demonstrated that *H. pylori* is able to up-regulate VEGF expression in gastric epithelial cells determining effects related to *vacA*-expression.

Recently, for the first time, Caputo *et al*^[13] showed that *H. pylori* up-regulated VEGF expression in gastric mucosa cells *in vitro* and that this effect was strictly *vacA*-dependent; and, interestingly, this result was not observed when using an isogenic mutant specifically lacking *vacA*. Moreover, *in vitro* and *in vivo* up-regulation of a number of EGF-related growth factors have also been reported^[15,16,49].

In our sufficiently large series of gastric cancer patients, a percentage of *H. pylori* infection was demonstrated, either as IgG-circulating level or as *cagA*/*vacA* DNA, which is in agreement with the previous studies^[50,51]. In addition, the neo-angiogenesis characteristics reported did not significantly differ from those of other series of gastric cancers^[7,52,53]. It is also possible to verify a clear relationship between p53, TP, VEGF and the clinicopathological characteristics considered in our series, further stressing the impact that angiogenesis has on tumor aggressiveness^[52,53]. In fact, TP expression and plasma VEGF levels were both associated with tumor size, while p53 expression was associated with metastatic status.

However, when addressing the main objective of our study, it is possible to demonstrate an association only between higher levels of plasma VEGF and high levels of IgG (Table 3). Conversely, *H. pylori*-infected tumors did not show p53, MVD, TP, VEGF-R1 characteristics which obviously differed from those without presence of

H. pylori infection. These results only partially agree with previous data^[17,54] which, however, were all obtained from experimental *in vitro* models, in some cases referring to mRNA analyses utilizing only quantitative molecular or immuno-enzymatic approaches^[14], therefore not exploiting morphological antigen tissue distribution. Furthermore, the increase of gene expression induced by the co-culture of gastric tumor cells with *H. pylori* has been reported to be generally modest with more evident positive modulation for angiogenic factors not investigated in the present study, such as interleukin-8^[14]. Caputo *et al*^[13] also recently suggested that *vacA*-induced up-regulation of VEGF expression could depend on the functionality of epidermal growth factor receptor (EGFR)-, mitogen-activated protein kinase (MAPK)- and COX-2-mediated pathways, the biological targets which are largely heterogeneous in human gastric cancer. In conclusion, our study seems to suggest that the relationship between the *H. pylori* toxic effect and angiogenic factors demonstrated *in vitro* could be influenced in human gastric tumor tissues by other key biological factors not considered in the present study.

A last comment regarding IgG and VEGF association concerns the blood of gastric cancer patients. The level of IgG antibodies has been suggested to be useful, not for diagnosis of infection, but for monitoring the outcome of *H. pylori* infection over time, and specifically the efficacy of therapies aiming to eradicate *H. pylori* infection. Thus, an elevated serum level before primary surgery for gastric cancer could be a signal of long-lasting and probably *H. pylori* infection resistant to antimicrobial therapy^[55].

The results of our study indicate that, from the angiogenic point of view, *H. pylori*-related gastric cancers do not differ from those in which exposure to the bacterium cannot be demonstrated. Different explanations for these findings can be proposed: an angiogenetic relationship, if any, could be only induced by a long-lasting *H. pylori* infection demonstrated by high IgG levels in the plasma; an alternative hypothesis might regard the ability of *H. pylori* to modulate angiogenesis only during early phase of disease genesis and progression to be then lost during the clinically evident disease phases. This hypothesis would concord with the presumed role that angiogenesis plays, especially during the extremely early phases of cancer^[9,17,56].

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-765G > C *COX-2* polymorphism may be a susceptibility marker for gastric adenocarcinoma in patients with atrophy or intestinal metaplasia

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considered as another susceptibility marker for gastric adenocarcinoma development in patients with atrophy or intestinal metaplasia.

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Abstract

AIM: To investigate the relationship between the -765G > C *COX-2* polymorphism and the development of different gastric lesions: atrophy or intestinal metaplasia and gastric adenocarcinoma.

METHODS: A cross-sectional study was performed involving 320 Portuguese individuals (210 without evidence of neoplastic disease, 73 patients with gastric adenocarcinomas and 37 with atrophy or intestinal metaplasia) using a PCR-RFLP method.

RESULTS: -765C allele was overrepresented in the patients with gastric adenocarcinoma (51%) when compared either with the control group (38%) or patients with atrophy or intestinal metaplasia (27%). Allele was found to be very common in our population (0.22), and a multivariate logistic regression analysis revealed nearly 3-fold increased risk for the progression to gastric adenocarcinoma in patients with atrophy or intestinal metaplasia carrying the -765C allele (OR = 2.67, 95% CI = 1.03-6.93; *P* = 0.04).

CONCLUSION: -765C carrier status should be

INTRODUCTION

Gastric adenocarcinoma mortality rates have been decreasing in Europe^[1], although, in Portugal it still remains one of the leading causes of cancer-related deaths (third in men and fourth in women). Portugal has one of the highest mortality rates of Europe and the highest in southern Europe, with values of 33.2 and 20.8 per 100 000/year in men and women, respectively^[2].

Cyclooxygenase (COX), also known as prostaglandin endoperoxide synthase, is a rate-limiting enzyme that converts free arachidonic acid into important prostanoids (PGs) and eicosanoids such as prostaglandin H₂^[3]. There are at least two isoforms of COX identified^[4]: *COX-1* is expressed constitutively in most cell types and is thought to be responsible for the maintenance of vascular homeostasis and gastroprotection^[3,4]; and, *COX-2*, the inducible isoform of the enzyme, undetectable in most cells is readily induced by bacterial lipopolysaccharide (LPS), cytokine, growth factors, mitogens and tumor promoters^[5-6].

Enhanced expression of *COX-2* has been observed in several forms of cancer^[7-14], including gastric cancer and precancerous tissues^[15-18]. *COX-2* over-expression plays an important role in the inhibition of apoptosis, tumor growth, angiogenesis, invasion and metastasis, which are considered to be important steps in cancer development^[3,15,16,19-24].

Single Nucleotide Polymorphisms (SNP) are the most common form of genetic variants of the human genome^[25], some of which might have functional effects on the susceptibility to the development of human cancers^[26-33] by modifying the transcriptional activation.

Several polymorphisms in *COX-2* have been identified so far. However, only a few seemed to have a functional effect on the transcription. Recently, Papafili *et al*^[34] described a new polymorphism in the promoter region of *COX-2*, characterized by a guanine (G) to cytosine (C) transition at position -765 (-765G > C). This polymorphism appears to disrupt a *Stimulatory protein 1* (Sp1) binding site, which is considered to be a positive activator of transcription and leads to a 30% reduction of the *COX-2* promoter activity *in vitro*^[34]. With this evidence, only few investigations have been done involving *COX-2* polymorphisms either in cancer related studies^[25,35-42] or other diseases^[34,43-45].

The aim of this study was to determine the allelic frequencies of the -765G > C *COX-2* polymorphism in a northern Portuguese population and to investigate its association with the development of gastric lesions, such as atrophic gastritis or intestinal metaplasia and gastric adenocarcinoma.

MATERIALS AND METHODS

Subjects

The -765G > C *COX-2* polymorphism was evaluated in a cross-sectional study performed in healthy individuals without clinical evidence of cancer ($n = 210$) and patients with known gastric lesions ($n = 110$), both from the northern region of Portugal attended at the Portuguese Institute of Oncology (Porto, Portugal).

The control group was formed by 75 females and 135 males (64%) with a median age of 51 years old. Patients were divided according to the type of lesion presented upon histopathological diagnosis after endoscopic multiple biopsies. Seventy three patients displayed lesions as severe as high-grade non-invasive neoplasia and intestinal type invasive gastric adenocarcinoma and 37 with lesions, such as atrophy or intestinal metaplasia that belong to a standardized follow-up since 2001^[46]. The three different groups are characterized in Table 1. The group of patients with gastric adenocarcinoma included 27 females and 31 males (53%) with a mean age of 54 years and, and the group of patients with atrophy or intestinal metaplasia included 21 females and 13 males (38%) with a mean age of 61 years.

All samples were obtained with the permissions of the individuals before their inclusion in the study after informed consent according to the Declaration of Helsinki.

Sample DNA extraction

Blood samples were collected with a standard venipuncture technique using EDTA containing tubes. Genomic DNA was extracted from peripheral blood leukocytes by a standard Salting-out protocol^[47].

Genotyping of -765G>C *COX-2* polymorphism

The analysis of the -765G > C polymorphism was

Table 1 Characteristics of the participants: age, gender and type and stage of lesions

	Control $n = 210$	Gastric adenocarcinoma $n = 73$	Atrophy or intestinal metaplasia $n = 37$
Age (mean \pm SD)	49.5 \pm 18.0	54.2 \pm 11.3	60.7 \pm 10.9
Male Gender n (%)	135 (64)	36 (49)	15 (40)
Atrophy n (%)	Na	Na	3 (8)
Complete IM n (%)	Na	Na	4 (11)
Incomplete IM n (%)	Na	Na	30 (81)

Na: Not applicable; Atrophic chronic gastritis without intestinal metaplasia.

performed by PCR-based restriction fragment length polymorphism (PCR-RFLP) as previously described^[43]. The primers used in the amplification were: CX2A (forward): 5'-ATT CTG GCC ATC GCC GCT TC-3' and CX2B (reverse) 5'-CTC CTT GTT TCT TGG AAA GAG ACG-3' (Metabion, Martinsried, Deutschland). The amplification conditions were 95°C during 10 min for the initial denaturation step, followed by 35 cycles of denaturation at 94°C (1 min), annealing at 59°C (1 min) and extension at 72°C (1 min). The final extension step consisted 10 min at 72°C. As a negative control PCR mix without DNA sample was used to ensure contamination free PCR product. Reaction products were digested with *Bsh1236I* restriction endonuclease (Fermentas, Vilnius, Lithuania) during 8 h at 37°C.

Results were observed in 3% agarose gel stained with ethidium bromide (Figure 1). Fragment sizes of 134 + 23 bp indicated a wild-type homozygous -756GG genotype, and an uncut fragment of 157 bp indicated the homozygous -765CC genotype. The presence of all the three bands (23, 134, and 157 bp) indicated a heterozygous -765GC genotype. The 23 bp fragment, resulting from the *Bsh1236I* restriction can not be distinguished from the primer-dimer band in the agarose gel. Analysis of genotypes was independently performed by two of the authors (C.P. and P.F). Cases with nonconcordant results between the two observers, or with the absence of a PCR product were rejected. Also a second PCR-RFLP analysis was performed in ten per cent of all samples to confirm the genotype.

Variables

Individual's age and gender, type of gastric lesion (gastric adenocarcinoma or atrophy or intestinal metaplasia) or its absence, and *COX-2* alleles (G, C).

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. Multivariate logistic regression analysis was used to estimate odds ratio (OR) and its 95% confidence interval (CI) as a measure of the association between Allele C carrier and the risk for the development of gastric lesion. Gender and age were included in multivariate analysis, and assessment for interaction was considered in the model.

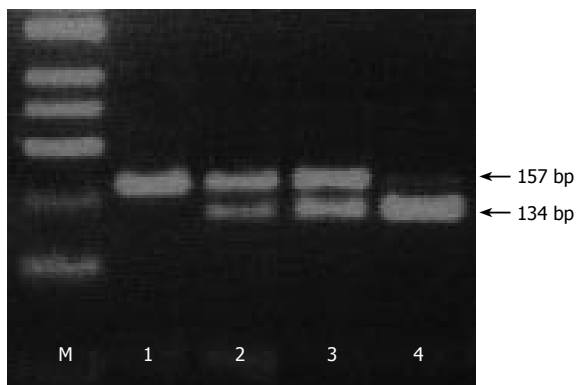


Figure 1 PCR-RFLP analysis of -765G > C *COX-2* polymorphism. M: 50 bp DNA ladder; 1: Homozygous -765CC genotype; 2, 3: Heterozygous -765GC genotype; 4: Homozygous -765GG genotype.

Table 2 Genotype distribution of *COX-2* -765G > C polymorphism

Genotype	Controls (<i>n</i> = 210)		Atrophy or intestinal metaplasia (<i>n</i> = 37)		Gastric adenocarcinoma (<i>n</i> = 73)
	<i>n</i> (%)	<i>P</i> ¹	<i>n</i> (%)	<i>P</i> ²	<i>n</i> (%)
GG	130 (62)	0.197	27 (73)	0.018	36 (49)
GC	67 (32)	0.357	9 (24)	0.046	32 (44)
CC	13 (6)	0.398	1 (3)	0.339	5 (7)
C carrier	80 (38)	0.197	10 (27)	0.018	37 (51)

¹ vs Atrophy or intestinal metaplasia group (chi-square test); ² vs Gastric adenocarcinoma group (chi-square test).

RESULTS

Allelic distribution of *COX-2* polymorphism

The distribution of -765G > C *COX-2* genotypes is shown in Table 2. The frequency of the -765GG, GC and CC genotypes were 62%, 32% and 6%, respectively in controls, 49%, 44% and 7% in patients with gastric adenocarcinoma and 73%, 24% and 3% in patients with atrophic gastritis or intestinal metaplasia. All genotypic distributions are in Hardy-Weinberg equilibrium ($P > 0.05$). -765C carriers were more frequently found among those with gastric adenocarcinoma ($P = 0.04$) than the other groups.

Risk estimate for associated lesions and invasive gastric adenocarcinoma

Table 3 describes the Odds Ratio for the development of atrophic gastritis or intestinal metaplasia and gastric adenocarcinoma. We found no statistically significant risk for the development of either atrophy or intestinal metaplasia (OR = 0.60, 95% CI = 0.28-1.31; $P = 0.20$) or gastric adenocarcinoma (OR = 1.67, 95% CI = 0.98-2.86; $P = 0.06$). Although the results for the development of gastric lesions were not statistically significant, we observed a possible protective role for -765C carriers, and when the same analysis was adjusted for age and gender by a multivariate logistic regression analysis this protective effect disappeared (OR = 0.95, 95% CI = 0.91-0.99; $P = 0.01$). In contrast, we observed a nearly 3-fold increased risk for the progression of atrophy or intestinal metaplasia

Table 3 Frequency distributions and Odds Ratio for risk of atrophy and intestinal metaplasia or gastric adenocarcinoma in -765 C carriers

Pathology	Genotype <i>n</i> (%)		OR (95% CI)		OR ² (95% CI)
	GG	C carrier			
Controls	130 (62)	80 (38)	1.00 (Reference)	1.00 (Reference) ¹	
Atrophy or intestinal metaplasia	27 (73)	10 (27)	0.60 (0.28-1.31)	0.95 (0.91-0.99) ¹	1.00 (Reference)
Gastric adenocarcinoma	36 (49)	37 (51)	1.67 (0.98-2.86)	1.45 (0.84-2.64) ¹	2.67 (1.03-6.93)

¹ OR adjusted for age and gender in a multivariate logistic regression analysis; ² OR adjusted for age and gender in a multivariate logistic regression analysis for the progression of atrophy or intestinal metaplasia into gastric cancer.

Table 4 C allele frequency and C carriers distribution in different countries

Country	<i>n</i> ¹	C allele frequency (%)	C carrier distribution (%)
America			
USA ^[39]	228	21	37
USA (African American) ^[39]	100	32	52
Europe			
Portugal (our study)	210	22	38
Italy ^[44]	864	28	50
UK ^[34]	454 (males)	14	25
Poland ^[43]	547	17	31
Australia			
Australia ^[45]	168	17	31
Asia			
Singapore ^[36]	1177	5	9
Japan ^[35]	241	2	5
China ^[18]	1270	2	4

¹ in control populations of the mentioned studies.

into gastric adenocarcinoma (OR = 2.67, 95% CI = 1.03-6.93; $P = 0.04$).

Furthermore, when we evaluated the distribution of gender in the two groups, atrophy or intestinal metaplasia and gastric adenocarcinoma, no statistically significant differences were observed ($P = 0.38$).

DISCUSSION

Enhanced expression of *COX-2* gene has been reported in several forms of cancer, including gastric precancerous and adenocarcinoma tissues^[15-18]. This evidence suggests a role of *COX-2* in the carcinogenesis pathway, such as in the inhibition of apoptosis, tumour growth, angiogenesis, invasion and metastasis^[3,15,16,19-24]. A -765G > C polymorphism on the promoter region of *COX-2* gene disrupts the Sp1 binding site^[34] that may alter the susceptibility to develop cancer^[36]. Our results revealed that C allele is extremely common (22%) in our population. Although only a few studies have been developed involving this *COX-2* polymorphism, the frequencies of the polymorphic variant seems to vary, especially among different ethnic populations (Table 4). These studies revealed that the C allele is

more frequent in Western countries, in Europe and America, than in Asian countries. Moreover, our results for the genotype frequencies are in concordance with other previously reported data in Caucasian populations.

-765G > C carriers and development of atrophic gastritis and intestinal metaplasia

In the present study, -765C carriers were slightly overrepresented in the control population (38%) when compared with patients with atrophy or intestinal metaplasia (27%). In fact, although not statistically significant, our results revealed a possible protective role for -765C carriers. Nevertheless, when adjusted for age and gender this protective role disappears (OR = 0.95, 95% CI = 0.91-0.99, $P = 0.01$) suggesting that this variant does not influence the development of gastric lesions such as atrophy or intestinal metaplasia. Although, the protective role is in agreement with previous studies, the small sample size may raise some statistical concerns to this observation. Papafili *et al*^[34] showed that the promoter activity of the -765C allele is reduced to about 30% when compared to the -765G. In addition, Ulrich *et al*^[38] revealed a marginal protection for the development of colorectal adenomatous and hyperplastic polyps when associated with the -765CC genotype. Thus, these results confirm the evidence that the depletion of the Sp1 binding site, considered a positive activator of COX-2 transcription, caused by the -765G > C transition, modifies the transcriptional activation of COX-2^[34].

-765G > C polymorphism and the development of gastric adenocarcinoma in patients with atrophic gastritis and intestinal metaplasia

Gastric cancer developed upon a multistep process from chronic active gastritis, gastric glandular atrophy (GA), intestinal metaplasia (IM), dysplasia and gastric cancer^[46]. In a recent work, it was suggested that COX-2 expression increases as it progresses from initial gastric lesions to gastric cancer, providing evidence that COX-2 might contribute to an early event in gastric carcinogenesis^[48]. Another approach attempt to understand the influence of the COX-2 -765G > C polymorphism has in the progression from atrophy or intestinal metaplasia lesions to gastric adenocarcinoma. We observed a nearly 3-fold increased risk of progression from gastric lesions into gastric cancer (OR = 2.67, 95% CI = 1.03-6.93; $P = 0.04$). This result is consistent with previously cancer-related studies that also revealed that -765C allele carriers had increased risk for the development of those diseases^[36,49]. More recently, Zhang *et al*^[42] described a 2-fold increased risk for the development of esophageal squamous cell carcinoma due to increased expression of COX-2 mRNA in -765G > C heterozygous. Although the exact molecular mechanism by which COX-2 polymorphism may affect the risk of gastric adenocarcinoma development is still unclear, studies in the COX-2 promoter revealed that COX-2 transcription is activated by E2 promoter binding factor 1 (E2F1)^[50], which is dependent on the transactivation and DNA-binding domains of E2F1^[51]. So the ability of this polymorphism to create an E2F binding site, essential for the expression of several genes^[43], might help us to understand why we

observed increased risk.

In conclusion, all these findings suggest that different physiological/pathological conditions, as well as cell type, could determine the influence that -765G > C COX-2 polymorphism has in the development of human diseases, by the modification of the binding sites for the transcription factors^[18]. The contribution of genetic polymorphism to the risk of gastric adenocarcinoma may be dependent on the population in study, as well on several environmental and dietary factors that influence that population. So, we hypothesize that each population has to evaluate its own genetic profile for cancer risk that may help to understand the geographic and racial differences reported for gastric adenocarcinoma^[52]. Furthermore, COX-2 polymorphisms may be involved in different individual drug response^[53,54] and may be explored as for in clinical trials to select those individual to be submitted to COX-2 inhibition. Moreover, the definition of a pharmacogenomic profile using molecular studies may help to the development of a personalized treatment or quimioprevention.

To the best of our knowledge, this is the first report that evaluates the -765G > C COX-2 polymorphisms and gastric adenocarcinoma development worldwide, which also considers the progression from gastric lesions, such as atrophy or intestinal metaplasia, to gastric adenocarcinoma. We theorise that once the lesions are installed, -765C carriers are at risk of progression into gastric adenocarcinoma. However, our results should be cautiously interpreted as they report a cross-sectional design. Therefore, we suggest that -765G > C polymorphism should be used in a large cohort study among patients with atrophy or intestinal metaplasia as a susceptibility marker for gastric adenocarcinoma and to confirm the real meaning of this genetic alteration in gastric adenocarcinoma development.

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***H pylori* infection among 1000 southern Iranian dyspeptic patients**

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Abstract

AIM: To describe the frequency of *H pylori* infection among 1000 southern Iranian dyspeptic patients.

METHODS: A prospective study was performed in a referral hospital in south of Iran from 1999 to 2005. One thousand dyspeptic patients (518 males, mean \pm SD age of 49.12 ± 12.82 years) consecutively underwent upper gastrointestinal endoscopy. Multiple gastric antral biopsy samples were taken from all patients for rapid urease test and histopathologic examination (96.9% satisfactory samples). Patients were considered *H pylori*-infected if one or both tests were positive.

RESULTS: Six hundred and seventy-one patients (67.1%, 95% confidence interval [CI]: 64.2%-70.0%) were *H pylori*-infected. *H pylori* positivity was significantly more frequent in patients with peptic ulcer disease (PUD) than in those with non-ulcer dyspepsia ($P < 0.001$). Male-to-female ratio for duodenal and gastric ulcers was 2.7:1 and 1.5:1, respectively. Moreover, the duodenal-to-gastric ulcer ratio was 1.95:1. The frequency of *H pylori* infection among those with endoscopic diagnosis of gastritis, duodenal ulcer, gastric ulcer, and normal mucosa was 70.1% (398/568), 86.2% (150/174), 71.9% (64/89), and 33.5% (54/161), respectively. *H pylori* infection, male sex, and older age were independently associated with PUD in multivariate analysis. *H pylori* positivity was associated with chronic gastritis, and chronic active gastritis with odds ratios of 34.21 (95% CI: 12.19%-96.03%) and 81.21 (95% CI: 28.85%-228.55%), respectively.

CONCLUSION: *H pylori* and PUD are highly frequent in dyspeptic patients from south of Iran. *H pylori* is a cardinal risk factor for chronic active or inactive gastritis.

INTRODUCTION

H pylori is a major cause of gastritis and peptic ulcer disease (PUD), and has been implicated in the development of gastric malignancy^[1-3]. The prevalence of *H pylori*, a worldwide infection, varies greatly among countries and among population groups within the same country^[4]. *H pylori* is highly prevalent in the developing countries^[5-11] and is common in 57%-91% of Iranian population^[5,12-14]. However, studies regarding *H pylori* prevalence in different regions of Iran, a country with a miscellaneous climate, are limited^[5,12-16]. Thus, we studied the frequency of *H pylori* infection among 1000 southern Iranian dyspeptic patients. Furthermore, we report *H pylori* association with different histopathologic and upper gastrointestinal endoscopy (UGIE) findings in this large number of patients.

MATERIALS AND METHODS

Subjects

A prospective study was performed in the Gastroenterology Division, Shahid-Mohammadi Referral Hospital, Bandarabbas, Iran from October 1999 to August 2005. One thousand consecutive patients (518 males, mean \pm SD age of 49.12 ± 12.82 years, range 13-89 years) with dyspepsia underwent UGIE. Those with age over 12 years, no prior gastric surgery, no active bleeding, and no consumption of antibiotics, bismuth preparations, or proton pump inhibitors in the 4 wk prior to UGIE were eligible to enter the study. All patients enrolled gave their informed consent and the study was performed according to the Declaration of Helsinki.

Methods

UGIE reported signs of gastritis, PUD, polyp, mucosal atrophy, and tumor. When PUD was present, concomitant gastritis was not mentioned in the study questionnaire. Moreover, when none of the above-mentioned pathologic

Table 1 Numbers and proportions of *H pylori* infected patients

	<i>n</i>	%	95% CI
Total	671/1000	67.1	64.2-70.0
Age (yr) ^a			
≤ 20	57/105	54.3	44.7-63.9
21-50	380/559	68.0	64.1-71.9
≥ 51	234/336	69.6	64.7-74.5
Sex			
Male	362/518	69.9	66.0-73.8
Female	309/482	64.1	59.8-68.4
UGIE finding ^b			
PUD	214/263	81.4	76.7-86.1
NUD	453/732	61.9	58.4-65.4
Tumor	4/5	80.0	40.8-100

PUD: Peptic ulcer disease; NUD: Nonulcer dyspepsia. ^a*P* < 0.05 between groups regarding *H pylori* infection; ^b*P* < 0.001 between PUD and NUD groups regarding *H pylori* infection.

features was present, the UGIE examination was considered normal. During UGIE, at least three biopsy specimens were taken from the antrum lesser curvature mucosa 3-4 cm proximal to the pylorus. One specimen was for rapid urease test (RUT). The RUT was monitored for color change up to 6 h after addition of the gastric tissue. The test was scored as positive if the color changed from yellow to red. The remaining two specimens were fixed in formaldehyde and submitted for histologic examination and HE staining. Nine hundred and sixty-nine antral mucosa biopsies (96.9%) were satisfactory for histopathological examination. One experienced pathologist, blinded to the results of RUT and UGIE, evaluated the coded samples. Thirty percent of coded samples (*n* = 291) were randomly evaluated by a second experienced pathologist. When the results were different (*n* = 11), the slides were discussed in joint sessions where a third pathologist was also present. The slides were observed under an optical microscope at several magnifications (including oil immersion). An increase in lymphocytes and plasma cells in the lamina propria characterized the gastritis as chronic. Activity in the context of chronic gastritis referred to the density of neutrophil polymorphs in the lamina propria, gastric pits, and surface epithelium. The presence of any pathology in at least one biopsy sample was considered a positive finding. The absence of abnormal findings in all specimens was regarded as normal. Detection of any *H pylori* was contemplated evidence of infection. Patients were considered *H pylori* positive if RUT and/or histology were/was positive. When the histologic sample was unsatisfactory, patient's of *H pylori* infection was determined by the RUT.

The frequency of *H pylori* was evaluated by means of cross tables regarding different age, sex, endoscopic and histopathologic diagnoses and tested by chi-square (χ^2) test and Fisher's exact test. Multivariate analysis was performed by entering sex, age groups, and *H pylori* status in a logistic regression model to identify the independent risk factors for endoscopic diagnoses of duodenal ulcer, gastric ulcer, and gastritis (normal endoscopy was set as a reference category). The same model was built to identify the independent risk factors for histopathologic diagnoses (normal mucosa was set as a reference category). For all

Table 2 Histological findings of satisfactory 969 antral biopsies

Histological finding	<i>n</i>	% (95% CI)
Normal mucosa	84	8.7 (6.9-10.4)
Chronic inactive gastritis	365	37.7 (34.6-40.7)
Chronic active gastritis	456	47.1 (43.9-50.2)
Atrophic changes	242	25.0 (23.6-27.7)
Intestinal metaplasia/malignancy	81	8.9 (7.1-10.7)
Glandular dysplasia	1	0.1 (-0.1-0.3)
Adenocarcinoma	4	0.4 (0.0-0.8)

point prevalences, 95% confidence intervals (CI) were calculated. Statistical significance was set at *P* < 0.05.

RESULTS

Six hundred and seventy-one patients (67.1%, Table 1) were *H pylori*-infected. PUD was found in 263 enrolled patients (26.3%, 95% CI: 23.6%-29.0%). Those with PUD were significantly more *H pylori*-infected than those with non-ulcer dyspepsia (NUD) (*P* < 0.001, Table 1). Fisher's exact test displayed that PUD was more frequent in male patients than in female patients (68.8%, 95% CI: 63.2%-74.5%) *vs* 31.2% (95% CI: 25.5%-36.8%) (*P* < 0.001). Male-to-female ratio for duodenal and gastric ulcers was 2.7:1 (127/47) and 1.5:1 (54/35), respectively. While the duodenal-to-gastric ulcer ratio was 1.9:1 (174/89), endoscopic signs of gastritis were evident in 568 (56.8%), polyp in 7 (0.7%), mucosal atrophy in 5 (0.5%), raised/thickened area in 5 (0.5%), and accompanying duodenal and gastric ulcers in 3 (0.3%) patients. Thus, 161 patients (16.1%) had normal endoscopic results. A histologic diagnosis of chronic active gastritis was assigned to 47.1% of biopsy samples. Table 2 summarizes the histopathological findings.

While *H pylori* was frequent in 33.5% of those with normal UGIE, 86.2% of those with duodenal ulcer were *H pylori*-infected (*P* < 0.001, Table 3). Those with histologic diagnosis of chronic active gastritis were significantly more *H pylori*-infected than those with chronic inactive gastritis (*P* < 0.001, Table 3). Table 3 summarizes the frequency of *H pylori* infection regarding different endoscopic and histopathologic findings. Multivariate logistic regression analysis proved *H pylori* positivity, older age, and male gender as predictors of duodenal ulcer, gastric ulcer, and gastritis (Table 4). The second model to identify the risk factors for histopathologic findings, had a 56.1% agreement between predicted and observed results and the amount of variance accounted for 21.4% (Cox & Snell) - 25.3% (Nagelkerke). This model suggested that *H pylori* positivity was associated with chronic gastritis, and chronic active gastritis with odds ratios (OR) of 34.21 (95% CI: 12.19%-96.03%) and 81.21 (95% CI: 28.85%-228.55%), respectively.

DISCUSSION

In the developing world, *H pylori* is a challenging health problem as 20% prevalence of *H pylori* infection among adolescents in the United States pales in comparison with infection rates exceeding 90% by five years of age in parts of the developing world^[17]. One study in northwest of

Table 3 *H. pylori* infection regarding endoscopic and histologic diagnoses *n* (%)

Diagnosis	<i>H. pylori</i> infection		
	Negative	Positive	Total
Endoscopic findings			
Normal	107 (66.5)	54 (33.5)	161
Gastritis	170 (29.9)	398 (70.1) ^b	568
Duodenal Ulcer	24 (13.8)	150 (86.2) ^{b,d}	174
Gastric Ulcer	25 (28.1)	64 (71.9) ^b	89
Histopathologic findings			
Normal mucosa	80 (95.2)	4 (4.8)	84
Chronic inactive gastritis	130 (35.6)	235 (64.4) ^f	365
Chronic active gastritis	87 (19.1)	369 (80.9) ^h	456
Atrophic changes	76 (31.4)	166 (68.6) ^f	242
Metaplasia/dysplasia/adenocarcinoma	15 (17.4)	71 (82.6) ^f	86

^b*P* < 0.001 *vs* normal endoscopic findings; ^d*P* < 0.001 *vs* gastritis and gastric ulcer; ^f*P* < 0.001 *vs* normal histopathologic findings; ^h*P* < 0.001 *vs* histopathologic findings and chronic inactive gastritis (Fisher's exact test).

Iran, a region with the highest mortality rate from gastric cancer throughout the country, reported that *H. pylori* infection occurs in 89.2% (883/990) of the residents^[5]. Other surveys in different age groups from various regions of the country reported that *H. pylori* infection occurs in 57%-91% of the study subjects^[12-14]. In this prospective survey, we report *H. pylori* infection in 67.1% of 1000 enrolled dyspeptic patients from south of Iran. Variation in study powers as well as ethnicity, place of birth, socioeconomic factors, diet, occupation, smoking, or alcohol consumption habits among study populations may be the reasons for erratic rates of *H. pylori* infection reported from the country^[6,18]. Similarly, *H. pylori* seems to be a health problem in the neighboring regions of Iran. In India, *H. pylori* is positive in 38 (56.7%) asymptomatic individuals and in 49 (61.3%) symptomatic individuals^[6]. In Saudi-Arabia, *H. pylori* is present in 54.9% of gastric biopsies from 488 dyspeptic patients^[7]. In Yemen, 82.2% of 275 dyspeptic patients are *H. pylori*-infected^[8]. In Jordan, *H. pylori* is frequent in 82% of 197 study subjects^[9]. In United Arab Emirates^[10] and in Kuwait^[11], 90.39% of 437 and 96.6% of 204 studied subjects are infected with *H. pylori*, respectively.

About a quarter of dyspeptic patients in this study were proved to have PUD. Nevertheless, in another large cohort of residents in northwest of Iran, the frequency of PUD is just 4.9%^[5]. This relatively low frequency of PUD might be due to the enrollment of unnecessarily dyspeptic subjects in the latter survey. PUD frequencies are divergent in reports from different countries. In a literature review by the American Gastroenterology Association^[19], 19 out of 41 studies report duodenal ulcer in $\geq 10\%$ of dyspeptic patients and the overall prevalence of PUD in these groups of symptomatic patients is $\geq 15\%$ in 21 studies. Duodenal ulcer was approximately twice as common as gastric ulcer in the present survey, which is in quite contrast to the 12:1 ratio reported from India^[6]. Moreover, *H. pylori* infection is significantly more frequent in PUD than in NUD patients. Regarding the significantly higher rate of *H. pylori* infection in those with duodenal (86.2%) and gastric (71.9%) ulcers in comparison with the subjects with normal endoscopic findings

Table 4 Multivariate logistic regression of selected model variables on endoscopic findings¹

Variable	B	SE	Wald test	P	Odds ratio (95% CI)
Duodenal ulcer					
Sex (male)	1.91	0.26	55.14	< 0.001	6.75 (4.08-11.17)
Age (in three ascending groups)	0.59	0.19	9.211	< 0.01	1.80 (1.23-2.63)
<i>H. pylori</i> (+)	2.54	0.28	79.21	< 0.001	12.66 (7.24-22.14)
Constant	-3.75	0.50			
Gastric Ulcer					
Sex (male)	1.34	0.28	22.13	< 0.001	3.83 (2.19-6.69)
Age (in three ascending groups)	0.65	0.22	8.49	< 0.01	1.91 (1.24-2.95)
<i>H. pylori</i> (+)	1.63	0.29	30.74	< 0.001	5.12 (2.87-9.11)
Constant	-3.45	0.56			
Gastritis					
Sex (male)	0.91	0.20	20.15	< 0.001	2.48 (1.67-3.68)
Age (in three ascending groups)	0.65	0.15	18.35	< 0.001	1.92 (1.42-2.58)
<i>H. pylori</i> (+)	1.54	0.19	61.81	< 0.001	4.65 (3.17-6.82)
Constant	-1.30	0.361			

¹Normal endoscopy was set as a reference category; agreement between predicted and observed results: 57.2%; the amount of variance accounted for 17.6% (Cox & Snell)- 19.4% (Nagelkerke).

(33.5%) (Table 3), also the significant association of *H. pylori* positivity with duodenal (OR: 12.66) as well as gastric ulcers (OR: 5.12) (Table 4), *H. pylori* can be introduced as an aetiological agent for PUD, thus strengthening prior findings^[10,20,21]. Furthermore, current evidence shows the cardinal role of *H. pylori* in the pathogenesis of PUD^[22-24]. In favor of the results of some studies^[25-27] and against the findings of others^[6,28,29], we found significantly more *H. pylori* infections in male than in female PUD subjects, but *H. pylori* was not significantly more prevalent in males. Regarding these two latter findings, one may deduce that *H. pylori* infection independently results in PUD in males more frequent than in females. The regression model also confirms this judgment as entering both sex and *H. pylori* status in the model showed an independent significant role of both factors in prediction of different endoscopic findings (Table 4). Nevertheless, despite more than a 50% agreement between the predicted and observed results of both models in this survey, outcomes should be carefully interpreted due to the limited factors entered into these models as other factors with a possible predictive role were beyond the scope of the current study and thus were not entered into the model.

In the present study, histologic findings of chronic active and inactive gastritis were frequent in about 85% of dyspeptic subjects, which is comparable with the previous 77.8% of chronic active gastritis in northwest of Iran^[5], 80.6% and 67% of chronic gastritis in Saudi-Arabia^[30] and India^[6], respectively. Similarly, *H. pylori* with a significantly higher frequency in those with chronic active and inactive gastritis compared to those with normal histology, showed a strong association with chronic active gastritis (OR: 81.21) and chronic inactive (OR: 34.21) gastritis, although more frequent in those with chronic active gastritis, which is suggestive of its causative role in chronic gastritis and gastritis activity. Despite some doubts^[31], *H. pylori* is globally believed to have a fundamental role in the pathogenesis of gastric cancer^[3,32,33]. Chronic *H. pylori* gastritis leads in more

than half of the affected subjects to a gradual loss of the glandular structures with its specialized cells and a collapse of the reticulin skeleton of the mucosa, a condition of atrophic gastritis^[34]. Indeed, the most common type of gastric cancer, the intestinal type, is preceded by chronic atrophic gastritis, which is 22%-37% prevalent in asymptomatic European adult subjects^[35]. In this survey, a quarter of satisfactory antral biopsies were proved to have atrophic changes in histology, about two thirds of which were associated with *H pylori* infection. Compared to our findings in south of Iran as well as those in the developed world^[35], northwest of Iran with a relatively higher frequency of atrophic changes in the antral biopsies of the sampled population (45.2%)^[5] might be at a higher risk of prevalence of gastric malignancies in the near future, an alarming condition that necessitates further investigations and thoughtful interventions.

In conclusion, *H pylori* and PUD are frequent in dyspeptic patients from south of Iran. *H pylori* infection, male sex, and older age are independently associated with PUD. *H pylori* is associated with chronic gastritis and even more with chronic active gastritis.

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Occurrence of cGMP/nitric oxide-sensitive store-operated calcium entry in fibroblasts and its effect on matrix metalloproteinase secretion

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CONCLUSION: NO/cGMP sensitive store-operated Ca^{2+} entry occurs in fibroblasts, and attenuates their adhesion potentials through its influence on MMP secretion.

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Key words: cGMP; Nitric oxide; Protein kinase G; Store-operated Ca^{2+} entry; Matrix metalloproteinase; Fibroblast

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Abstract

AIM: To examine the existence of Nitric oxide/cGMP sensitive store-operated Ca^{2+} entry in mouse fibroblast NIH/3T3 cells and its influence on matrix metalloproteinase (MMP) production and adhesion ability of fibroblasts.

METHODS: NIH/3T3 cells were cultured. Confocal laser scanning microscopy was used to examine the existence of thapsigargin-induced store-operated Ca^{2+} entry in fibroblasts. Gelatin zymography and semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) were employed to detect the involvement of $[\text{Ca}^{2+}]_i$ and NO/cGMP in MMP secretion. The involvement of NO/cGMP-sensitive Ca^{2+} entry in adhesion was determined using matrigel-coated culture plates.

RESULTS: 8-bromo-cGMP inhibited the thapsigargin-induced Ca^{2+} entry in 3T3 cells. The cGMP-induced inhibition was abolished by an inhibitor of protein kinase G, KT5823 (1 $\mu\text{mol/L}$). A similar effect on the Ca^{2+} entry was observed in 3T3 cells in response to a NO donor, (\pm)-S-nitroso-N-acetylpenicillamine (SNAP). The inhibitory effect of SNAP on the thapsigargin-induced Ca^{2+} entry was also observed, indicating NO/cGMP-regulated Ca^{2+} entry in 3T3 cells. Results of gelatin zymography assay showed that addition of extracellular Ca^{2+} concentration induced MMP release and activation in a dose-dependent manner. RT-PCR also showed that cGMP and SNAP reduced the production of MMP mRNA in 3T3 cells. Experiments investigating adhesion potentials demonstrated that cGMP and SNAP could upgrade 3T3 cell attachment rate to the matrigel-coated culture plates.

INTRODUCTION

Members of matrix metalloproteinase (MMP) family have been broadly implicated in both physiological and pathophysiological tissue remodeling^[1], and play a key role in tumor invasion and metastasis. Since degradation of extracellular matrix (ECM) components by MMP is critical for tumor cell invasion and metastasis^[2,3], MMP-2 degrades type IV collagen, a major component of the basement membrane because of its activity. Recent studies have revealed that tumor cells utilize MMP-2 produced by neighboring stromal cells including fibroblasts rather than by tumor cells themselves^[4,5] for tumor progression, invasion, and metastasis, and that tumor cells can stimulate MMP production by stromal cells via soluble factors such as cytokines^[6-9] or through cell-cell interaction mediated by cell adhesion molecules such as CD147^[10,11].

Studies have proved that stromal cells mainly release MMP around tumor cells, and that calcium is a second intracellular messenger which mediates a wide range of cellular responses. These studies aroused our interest in whether calcium ions are involved in MMP secretion. In non-excitable cells, the influx of Ca^{2+} is a biphasic process, which consists of an initial transient phase followed by a large and sustained phase. Under Ca^{2+} -free conditions, the response is only a small and transient rise in $[\text{Ca}^{2+}]_i$, which should reflect the release of intracellular Ca^{2+} stores. When extracellular Ca^{2+} is reintroduced in the absence of the agonist, there is a large rise in $[\text{Ca}^{2+}]_i$. Since the depletion of intracellular Ca^{2+} stores is apparently the single

mechanism at work under such a circumstance, it has been hypothesized that the Ca^{2+} entry is activated by the depletion of Ca^{2+} stores and this Ca^{2+} entry is thus called store-operated Ca^{2+} entry (SOC)^[12].

Nitric oxide (NO) is also known to inhibit Ca^{2+} entry through L-type Ca^{2+} channels (LTCC) in SMCs via cGMP-dependent mechanisms or via membrane hyperpolarization due to cGMP-dependent activation of Ca^{2+} -dependent K^{+} channels. Besides the direct effects of NO on Ca^{2+} entry, it can also activate heme-containing soluble guanylyl cyclase (sGC) and catalyze the production of cGMP from GTP, thus playing a role in NO/cGMP/protein kinase G (PKG) pathway. Although many reports have described the regulating mechanisms on the calcium channels, they all focused on the end result-- increase in $[\text{Ca}^{2+}]_i$. Thus further investigation is certainly required to clarify the role of NO/cGMP in SOC and MMP secretion.

The aim of the present study was to demonstrate the existence of NO/cGMP-regulated SOC in NIH/3T3 cells and to further explore the influence of store-operated Ca^{2+} entry on MMP secretion and adhesion potential in NIH/3T3 cells.

MATERIALS AND METHODS

Cell culture and reagents

NIH/3T3 cells (CRL-1658, obtained from American Tissue Culture Collection, ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 100 mL/L fetal calf serum. Cultures were maintained at 37°C in a humidified incubator under an atmosphere of 950 mL/L air and 50 mL/L CO_2 . 8-bromo-cGMP, (\pm)-*S*-nitroso-*N*-acetylpenicillamine (SNAP), *N*-methyl-(8*R*,9*S*,11*S*)-(-)-9-methoxy-9-methoxycarbonyl-8-methyl-3,10-dihydro-8-11-epoxy-1*H*, 8*H*, 11*H*-2,7*b*, 11*a*-triazadibenzo(a,g)cycloocta(cde) trinden-1-one (KT5823), N^G -nitro-L-arginine methyl ester (L-NAME) and thapsigargin were obtained from Calbiochem (La Jolla, CA). DMEM and fetal bovine serum were purchased from Life Technologies, Inc. Fluo3/AM was obtained from Molecular Probes, Inc. (Eugene, OR). One Step RNA PCR kit (AMV) was purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. MMP-2 primers (Accession no. NM008610; 5'ACCATCGAGACCATGCGG3', 5'CTCCCCCAACACCAGTGC3', 334 bp), MMP-9 primers (Accession no. NM013599; 5'TTCTGCCCTACCCGAGTGG3', 5'CATAGTGGGAGGTGCTGTCGG3', 426 bp), β -actin primers (5'CTCACTGTCCACCTTCCAG3', 5'CGACCATCTCCTCTTAGG3', 494 bp) were obtained from SaiBaiSheng Co. Trizol for total RNA isolation was from Life Technologies, Inc. Matrigel was obtained from Becton Dickinson Laboratory (Bedford, MA). Other reagents were from Sigma-Aldrich (St. Louis, MO).

Measurement of $[\text{Ca}^{2+}]_i$ in single cells by confocal laser scanning microscopy

After an overnight attachment, the cells were rinsed in 0.01 mol/L PBS and loaded with 5 $\mu\text{mol/L}$ Fluo3/AM for 45 min in dark at 37°C in normal PBS (NPBS) containing

2 mmol/L CaCl_2 , pH 7.4. The cells were then washed and resuspended in NPBS. To start the experiment, the cells were pretreated with 4 $\mu\text{mol/L}$ thapsigargin for 2 min. Then the cells were washed with and maintained briefly in PBS containing no Ca^{2+} and 2 mmol/L EGTA. Unless stated otherwise, the cells were pretreated with or without chemicals (i.e. 8-bromo-cGMP, KT5823, SNAP, L-NAME or NiCl_2) for 1-2 min. The fluorescent signal of cytoplasmic calcium ion concentration in 3T3 cells was determined by fluorescence using Bio-Rad MRC 1024 visible light CLSM (Bio-Rad, Hercules, CA) and argon (488/526 nm) laser light.

Gelatin zymography

The conditioned media were collected by centrifugation, concentrated and dialyzed. The dialyzed samples containing an equal amount (20 μg) of total protein were mixed with the sample buffer, incubated in water bath (about 55°C) for 3-5 min and loaded onto the zymographic 100 g/L SDS-polyacrylamide gel containing gelatin (1g/L) and run under standard conditions (2 h at constant voltage of 100V). Afterwards, the gels were washed once with 50 mmol/L Tris (pH 7.4), containing 25 mL/L Triton X-100 for 30 min, and twice with 50 mmol/L Tris (pH 7.4). Gels were then incubated for 16-18 h in 50 mmol/L Tris (pH 7.5), 0.15 NaCl, 10 mmol/L CaCl_2 , 1 mL/L Triton X-100 and 0.2 mg/L sodium azide. Finally, the gels were stained with Coomassie blue and washed with 75 mL/L acetic acid containing 200 mL/L methanol. The gels were subjected to densitometric analysis to quantitate the gelatinase activity by obtaining volumograms on a photo documentation system from UVItect (Cambridge, UK) using the UVIchrom acquisition software.

Semiquantitative reverse transcriptase-polymerase chain reaction

Total RNA was extracted from cGMP-pretreated 3T3 cells using Trizol agents. First-strand cDNA synthesis was performed using 1 μg of total RNA, 40 MU/L of RNase inhibitor, 20 MU/L sense and anti-sense primers of MMP-2, MMP-9 and β -actin, 10 mmol/L dNTP mixture, 25 mmol/L MgCl_2 , 5 MU/L AMV Rtae XL, 5 MU/L AMV-optimized Taq. MMP-2, MMP-9 and β -actin primers were reported in Material section. According to One Step RNA PCR kit (AMV) instructions, the following conditions were used for MMP-2: 28 cycles of PCR amplification, denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min; MMP-9: 30 cycles of PCR amplification, denaturation at 94°C for 50 s, annealing at 60°C for 1 min, and extension at 72°C for 2 min; β -actin: 23 cycles of PCR amplification, denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 1 min. Amplification was carried out in a GeneAmp 2400 PCR system (Perkins-Elmer, Foster City, CA). PCR products were resolved on 25 g/L agarose gel in the presence of 0.6 mg/L ethidium bromide. The intensities of the cDNA bands for each protein were normalized to β -actin band intensities. Images of the ethidium bromide (EB)-stained agarose gels were acquired with a digital Kodak camera (Eastman Kodak Company,

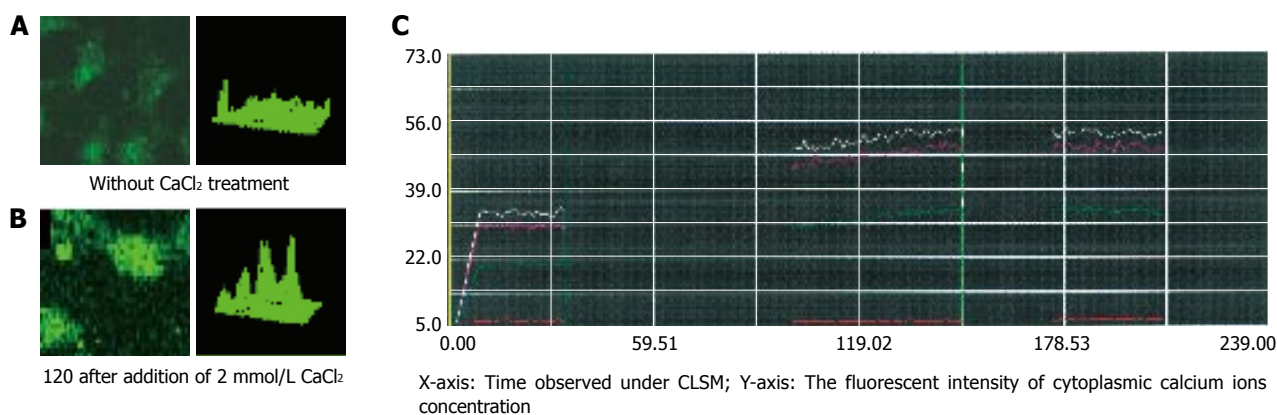


Figure 1 Tg-induced intracellular calcium ion concentration changes in 3T3 cells. **A:** The fluorescent intensity in 3T3 cells was 25-30 in Ca^{2+} -free and 2 mmol/L EGTA-containing medium after treatment with 4 $\mu\text{mol/L}$ Tg; **B:** The fluorescent intensity rose to 50-55 in 3T3 cells after adding 2 mmol/L CaCl_2 ; **C:** Similar intracellular free Ca^{2+} concentration dynamic changes in 3T3 cells and Tg-induced elevation of $[\text{Ca}^{2+}]_i$ in 3T3 cells 120 min after treatment.

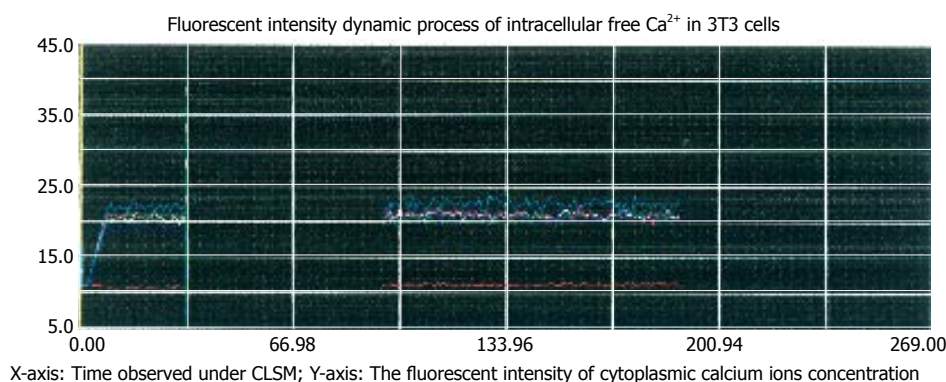


Figure 2 Inhibitory effect of Ni^{2+} on thapsigargin-induced rise in $[\text{Ca}^{2+}]_i$. The cells were incubated in NPBS with 4 $\mu\text{mol/L}$ thapsigargin and 3 mmol/L NiCl_2 for 90 s, then the media were replaced with respective media containing 2 mmol/L CaCl_2 without EGTA, no significant change of $[\text{Ca}^{2+}]_i$ occurred after 120 s.

Rochester, NY, USA) and quantification of the bands was performed by imaging analysis software from Kodak.

Cell adhesion assay

The wells of 96-well culture plates were coated with matrigel at a concentration of 5 mg/L and incubated at 4°C overnight. The coated wells were blocked with PBS containing 20 g/L bovine serum albumin for 30 min and then washed with PBS. Cell suspension in serum-free medium containing 1 g/L bovine serum albumin was added to the wells (2×10^4 /well) and incubated at 37°C in 50 mL/L CO_2 for 30-60 min with or without test agents (8-bromo-cGMP or SNAP). After the medium and nonattached cells were removed, 2 g/L crystal violet was added for 10 min. The plate was gently washed with tap water and dried in air for 24 h. Then 0.1 mL of 50 g/L SDS with 500 mL/L ethanol was added for 20 min, the plate was read at 540 nm on an ELISA reader (Microplate Co., EL311SX).

Statistical analysis

Data were expressed as mean \pm SD. Intracellular Ca^{2+} fluorescence ratio and percentages of attached cells were estimated with Student's *t*-test or analysis of variance followed by Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

RESULTS

Thapsigargin-induced store-operated Ca^{2+} entry in fibroblast NIH/3T3 cells

Thapsigargin was used to deplete intracellular Ca^{2+} stores and induce Ca^{2+} entry from extracellular space. After treatment with 4 $\mu\text{mol/L}$ thapsigargin in Ca^{2+} -free and 2 mmol/L EGTA-containing medium for 2 min, the addition of 2 mmol/L CaCl_2 induced a rise in $[\text{Ca}^{2+}]_i$ about 120 later (Figure 1). The thapsigargin-induced elevation of $[\text{Ca}^{2+}]_i$ was completely blocked by Ni^{2+} (3 mmol/L), a potent blocker of Ca^{2+} entry that competes for Ca^{2+} -binding sites in 3T3 cells (Figure 2), confirming the presence of capacitive Ca^{2+} entry in this cell type.

Effect of NO/cGMP/PKG signal pathway on store-operated Ca^{2+} entry

It has been reported that capacitive Ca^{2+} entry in hepatoma cells could be inhibited by cGMP, an activator of protein kinase G (PKG), and enhanced by KT5823, an inhibitor of PKG^[13-15]. The present study examined the effect of cGMP and KT5823 on the capacitive Ca^{2+} entry in 3T3 cells. The $[\text{Ca}^{2+}]_i$ fluorescence ratios were obtained in cells without chemical treatment (control) or treated with 2 mmol/L cGMP or 1 $\mu\text{mol/L}$ KT5823, respectively. Results showed that 2 mmol/L cGMP inhibited the Ca^{2+}

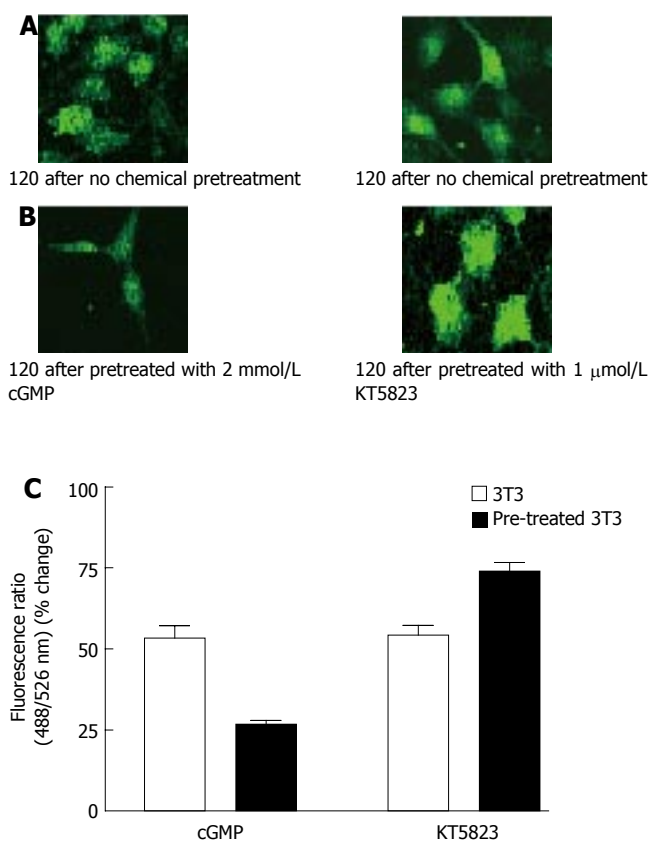


Figure 3 Effect of 8-bromo-cGMP and KT5823 on thapsigargin-induced Ca^{2+} influx. **A:** 3T3 cells were incubated in NPBS with 4 $\mu\text{mol/L}$ thapsigargin for 2 min, then 2 mmol/L CaCl_2 was added into the medium; **B:** 3T3 cells were incubated in NPBS with 4 $\mu\text{mol/L}$ thapsigargin for 2 min, 8-bromo-cGMP (2 mmol/L) or KT5823 (1 $\mu\text{mol/L}$) was introduced 1 min prior to the measurement, then 2 mmol/L CaCl_2 was added into the medium; **C:** Effects of cGMP and KT5823 on thapsigargin-induced Ca^{2+} influx. The Ca^{2+} entry fluorescence ratios were obtained in cells without chemical treatment (control) or treated with 2 mmol/L 8-Br-cGMP (cGMP) or 1 $\mu\text{mol/L}$ KT5823. The cells were incubated in NPBS with 4 $\mu\text{mol/L}$ thapsigargin for 2 min prior to the chemical treatment. One mmol/L CaCl_2 was introduced to the medium to obtain the fluorescent signal of Ca^{2+} entry. The values are the mean \pm SE ($n = 4-6$).

entry with $25.3\% \pm 1.3\%$ inhibition compared with 3T3 cells not pretreated ($53.0\% \pm 4.4\%$) ($P < 0.05$, Figure 3C). KT5823 (1 $\mu\text{mol/L}$) stimulated the Ca^{2+} entry by $72.3\% \pm 3.9\%$ ($P < 0.05$, Figure 3C).

NO is an important intracellular signal molecule that activates soluble guanylyl cyclase to synthesize cGMP^[16]. The present study was carried out to investigate the involvement of NO in regulation of the capacitive Ca^{2+} entry using a NO donor (SNAP) and L-NAME (a specific NOS inhibitor) to trigger production of endogenous cGMP. Results showed that SNAP also inhibited the thapsigargin-induced Ca^{2+} influx, $26.7\% \pm 6.4\%$ inhibition was observed at 100 $\mu\text{mol/L}$ SNAP compared with control ($48.1\% \pm 5.2\%$) ($P < 0.05$, Figure 4C). L-NAME (200 $\mu\text{mol/L}$) could excite the thapsigargin-induced Ca^{2+} influx in 3T3 cells too, $71.6\% \pm 3.9\%$ stimulation was observed in 3T3 cells compared with control ($49.6\% \pm 5.8\%$) ($P < 0.05$, Figure 4C).

Effect of SOC on MMP mRNA expression

The expression and release of MMPs (such as MMP-2 and MMP-9) work through kinase signaling pathways^[17-19],

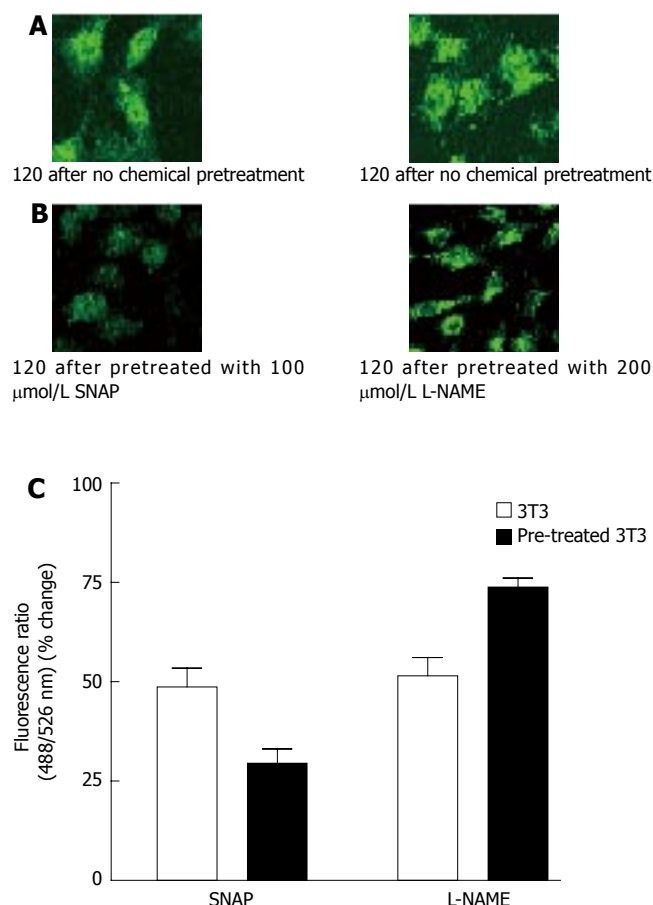


Figure 4 Effect of SNAP and L-NAME on thapsigargin-induced Ca^{2+} influx. **A:** 3T3 cells were incubated in NPBS with 4 $\mu\text{mol/L}$ thapsigargin for 2 min, then 2 mmol/L CaCl_2 was added into the medium; **B:** 3T3 cells were incubated in NPBS with 4 $\mu\text{mol/L}$ thapsigargin for 2 min, 100 $\mu\text{mol/L}$ SNAP or 200 $\mu\text{mol/L}$ L-NAME was introduced 1 min prior to the measurement, then 2 mmol/L CaCl_2 was added into the medium; **C:** Ca^{2+} entry fluorescence ratios were obtained in cells without chemical treatment (control) or treated with 100 $\mu\text{mol/L}$ SNAP or 200 $\mu\text{mol/L}$ L-NAME. The values are the mean \pm SE ($n = 4-6$).

like NO-cGMP- Ca^{2+} signaling pathway^[20-22]. The present study tested the involvement of SOC in regulating MMP release *in vitro*. RT-PCR showed that synthesis of MMP-2 and MMP-9 mRNA in cGMP-pretreated 3T3 cells was less than that in 3T3 cells not pretreated. Gelatin zymography showed that production of MMP-2 and MMP-9 in 3T3 medium culture was more than that in cGMP- pretreated 3T3 medium culture (Figure 5).

Involvement of $[\text{Ca}^{2+}]_i$ and NO/cGMP in MMP secretion

Zymography results showed that the increasing extracellular calcium concentrations (0, 0.09, 0.4, 0.8, 1.2 mmol/L) enhanced MMP-2 and MMP-9 secretion in a dose-dependent manner (Figure 6). In the present study, 4 $\mu\text{mol/L}$ thapsigargin was used to deplete the intracellular Ca^{2+} store in order to induce Ca^{2+} entry. Zymography results showed pretreatment with 8-bromo-cGMP (2 mmol/L) or SNAP (200 $\mu\text{mol/L}$) significantly reduced the production of MMP-2 and MMP-9 in 3T3 cells, and 1 $\mu\text{mol/L}$ KT5823 or 100 $\mu\text{mol/L}$ L-NAME enhanced the production of MMP-2 and MMP-9 in 3T3 cells. These results indicated that NO/cGMP sensitive SOC was

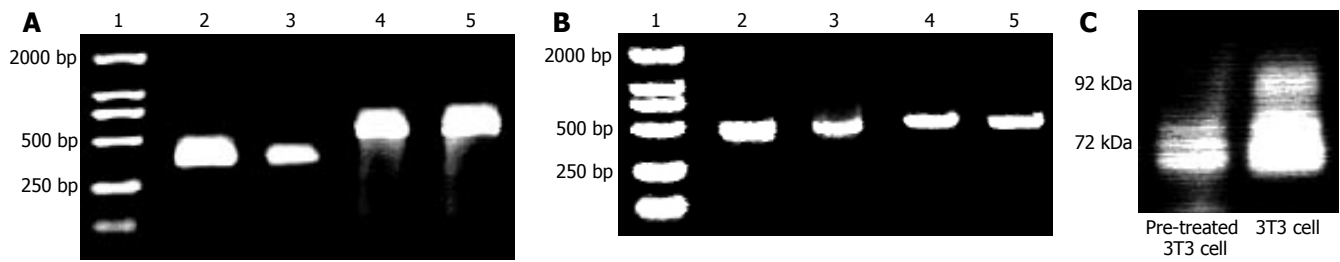


Figure 5 Expression of MMP in pretreated and not pretreated 3T3 cells. **A:** RT-PCR results of MMP-2 mRNA in both cells (1: DL2000 marker; 2: MMP-2 cDNA of 3T3 cells; 3: MMP-2 cDNA of 8-Br-cGMP (2 mmol/L) pre-treated 3T3 cells; 4: β -actin cDNA of 3T3 cells; 5: β -actin cDNA of pre-treated 3T3 cells); **B:** RT-PCR results of MMP-9 mRNA in pretreated and not pretreated 3T3 cells (1: DL2000 marker; 2: MMP-9 cDNA of 3T3 cells; 3: MMP-9 cDNA of 8-Br-cGMP (2 mM) pre-treated 3T3 cells; 4: β -actin cDNA of 3T3 cells; 5: β -actin cDNA of pre-treated 3T3 cells); **C:** Gelatin zymography showing constitutive secretion of MMP-2 (72 kDa) and MMP-9 (92 kDa) into serum-free media by pretreated and not pretreated 3T3 cells.

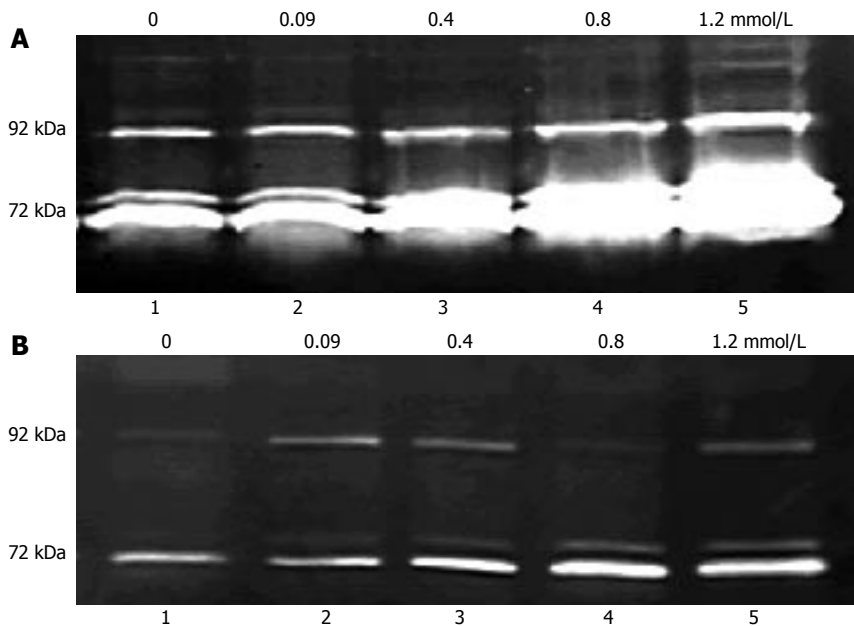


Figure 6 Dose-dependent effect of extracellular Ca^{2+} on MMP release. 8-bromo-cGMP (2 mmol/L) pre-treated and not pretreated 3T3 cells were plated in media with serial doses of Ca^{2+} (0, 0.09, 0.4, 0.8, 1.2 mmol/L) and incubated for 16–18 h, 200 μL conditioned media was used as sample. The 72 kDa gelatinolytic bands corresponded to the MMP-2, the 92 kDa band to MMP-9. **A:** 3T3 cell mean ratio of 72 kDa in groups 1–5 was 0.198 ± 0.094 , 0.287 ± 0.114 , 0.486 ± 0.079 , 1.247 ± 0.089 and 2.487 ± 0.179 , respectively ($n = 4$ –6); **B:** 8-bromo-cGMP (2 mmol/L) pre-treated 3T3 cell mean ratio of 72 kDa in groups 1–5 was 0.104 ± 0.084 , 0.126 ± 0.086 , 0.257 ± 0.102 , 0.490 ± 0.173 and 0.784 ± 0.187 , respectively ($n = 4$ –6).

involved in MMP secretion of fibroblasts (Figure 7).

Involvement of NO/cGMP-sensitive Ca^{2+} entry in adhesion

We used matrigel-coated plates as models for investigation of possible signaling pathways involved in extracellular matrix-induced metastasis. Matrigel is a soluble basement membrane preparation containing almost all extracellular matrix components^[23]. The present study was carried out with matrigel as an adhesion substratum. A significant difference in the amount of cells attached to the matrigel-coated plates was observed in 3T3 cells pre-treated and not pre-treated with cGMP (2 mmol/L) for 30 min ($71.4\% \pm 0.5\%$ vs $44.7\% \pm 2.5\%$) ($P < 0.05$). Pre-treatment with SNAP (200 $\mu\text{mol/L}$) showed that the percent of attached and non-attached 3T3 cells was $64.7\% \pm 1.2\%$ and $46.3\% \pm 2.0\%$, respectively ($P < 0.05$, Figure 8).

DISCUSSION

Collagenolytic degradation of endothelial and parenchymal basement membranes is a necessary step in the process of tumor invasion and angiogenesis. Neutral MMP-2 and MMP-9 mainly secreted locally by stromal cells, degrade the basement membrane and extracellular matrix collagens.

However, little is known about the signaling pathways that regulate the production of these enzymes. Previous studies have demonstrated that transforming growth factor- β 1 (TGF- β 1) plays a role in transmembrane signal transduction of MMP-2 regulation^[24]. The present study described a role of calcium mobilization in the regulation of MMP expression.

The present study demonstrated that the existence of NO/cGMP-sensitive store-operated Ca^{2+} entry in mouse fibroblast NIH/3T3 cells, which is allergic to NO/cGMP. The thapsigargin-induced SOC appears to be evidenced by the magnitude of the Ca^{2+} entry and its blockage by Ni^{2+} , cGMP or SNAP inhibits the thapsigargin-induced Ca^{2+} entry in 3T3 cells and can be reversed by KT5823 (highly specific PKG inhibitor), indicating a negative regulatory mechanism involving NO/cGMP *via* a PKG-dependent pathway.

Characterization of the regulation of interstitial collagenase (MMP-1) and stromelysin (MMP-3) indicates that there are at least two known regulatory arms for the secretion of these MMPs. Treatment with TGF- β 1 inhibits the production of these MMP genes through TIE, a novel CIS-acting transcriptional element^[25,26], whereas phorbol ester-mediated stimulation of transcription is

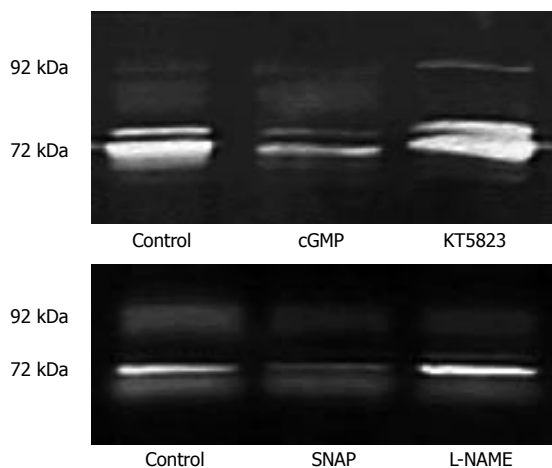


Figure 7 cGMP/KT5823 and SNAP/L-NAME affecting MMP release 3T3 cells via Tg-induced SOC. 3T3 cells were incubated in NPBS with 4 $\mu\text{mol/L}$ thapsigargin for 2 min. Each kind of chemical reagents (2 mmol/L cGMP, 1 $\mu\text{mol/L}$ KT5823, 100 $\mu\text{mol/L}$ SNAP or 200 $\mu\text{mol/L}$ L-NAME) was introduced 1 min prior to the measurement, then 2 mmol/L CaCl_2 was added into each medium and incubated for 16-18 h. The 72 kDa gelatinolytic band (MMP-2) was obtained in cells without chemical treatment (control) or treated with cGMP, KT5823, SNAP, L-NAME.

through phorbol ester-responsive elements (TREs) and AP-1 sites^[27,28]. Regulation of matrix metalloproteinases has also been linked to the second cAMP messenger signal transduction pathway^[29,30]. Several investigators have demonstrated the induction of MMP-1 production in response to cAMP either directly or after cell stimulation with cytokines that signal their effects through cAMP^[29,31]. However, in contrast to MMP-1 and MMP-3, neither MMP-2 nor MMP-9 has TIE or cAMP-regulated elements defined in their 5' sequences^[27,32]. The TRE mechanism has been implicated in the regulation of MMP-9 but not MMP-2^[27]. AP-1 sites have not been documented for MMP-2, although AP-1 and AP-2 sites are in the upstream of MMP-9^[27,32,33]. It was reported that there is a NF- κB binding site in the upstream sequence of MMP-9^[34]. Besides the factors influencing MMP secretion, calcium-activated signal transduction steps are also a common thread underlying important signal transduction pathways, including protein kinase C, phosphoinositide metabolism, and generation of arachidonic acid. However, few calcium-mediated transcriptional pathways have been identified for MMP secretion.

The present study was to identify NO/cGMP-sensitive production and activation of MMP through SOC. In our study, adding extracellular Ca^{2+} concentration induced release and activation of MMP (MMP-2, MMP-9) in a dose-dependent manner, cGMP and SNAP reduced MMP secretion in 3T3 cells. RT-PCR displayed that MMP-2 and MMP-9 mRNA synthesis in cGMP- pretreated 3T3 cells was significantly less than that in non cGMP-pretreated 3T3 cells. Tumor invasion is greatly dependent on the permissive action of the microenvironment. One critical factor is the production of proteolytic enzymes involved in the degradation and remodeling of ECM. Among these enzymes, MMPs represent a large family playing a key role in cell proliferation, angiogenesis, tumor invasion and metastasis^[6]. These enzymes principally degrade the ECM components and attenuate cell-cell adhesion ability so as to

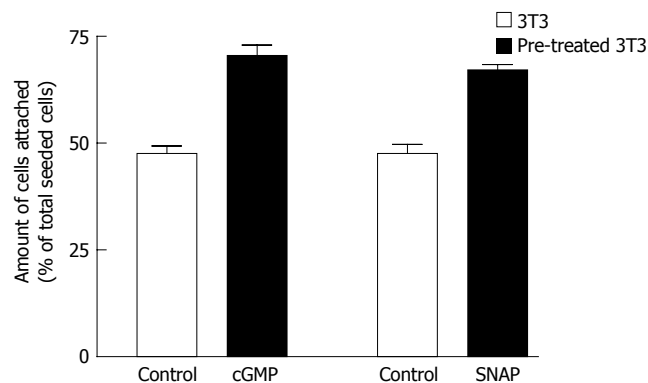


Figure 8 Effects of cGMP or SNAP on adhesion potentials of 3T3 cells to matrigel by adhesion assay. 3T3 cells pretreated with 4 $\mu\text{mol/L}$ Tg were suspended in serum-free medium supplemented with no chemical reagent (control) or cGMP (2 mmol/L) or SNAP (200 $\mu\text{mol/L}$). 2 mmol/L CaCl_2 was added to induce SOC, then seeded into the matrigel (5 mg/L)-coated wells. After incubation for 30 min at 37°C, the percentage of adhered cells was determined using colorimetric crystal violet assay. The values are the mean \pm SE ($n = 6-8$).

promote tumor invasion and metastasis.

Our experiments investigating adhesion potentials demonstrated that the attachment rate of 3T3 cells living in high extracellular Ca^{2+} concentration of the matrigel-coated culture plates was lower than that in low extracellular Ca^{2+} concentration of the matrigel-coated culture plates, suggesting that increased cellular Ca^{2+} concentration attenuates the adhesion potentials in fibroblasts by enhancing MMP secretion. The attachment rate could be upgraded by cGMP and SNAP in 3T3 cells, indicating that interference with NO/cGMP/PKG pathway sensitivity of fibroblasts reduces SOC and inhibits MMP secretion at a considerable level, thus strengthening the adhesion potential of fibroblasts and decreasing the chance of HCC metastasis.

In conclusion, store-operated Ca^{2+} channels exist in mouse fibroblast cells and are involved in MMP secretion. NO/cGMP/PKG pathway negatively regulates this Ca^{2+} entry. Increased extracellular Ca^{2+} concentration may promote degradation of ECM components by increasing MMP secretion and attenuating fibroblast adhesion potential.

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CLINICAL RESEARCH

Adiponectin and its receptors in rodent models of fatty liver disease and liver cirrhosis

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receptors was only found in liver cirrhosis.

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Abstract

AIM: To determine circulating and hepatic adiponectin in rodents with fatty liver disease or liver cirrhosis and investigate expression of the adiponectin receptors AdipoR1 on the mRNA and protein level and AdipoR2 on the mRNA level.

METHODS: Fat fed rats were used as a model for fatty liver disease and bile duct ligation in mice to investigate cirrhotic liver. Expression of AdipoR1 and AdipoR2 mRNA was determined by real time RT-PCR. AdipoR1 protein was analysed by immunoblot. Adiponectin was measured by ELISA.

RESULTS: Systemic adiponectin is reduced in fat-fed rats but is elevated in mice after bile duct ligation (BDL). Hepatic adiponectin protein is lower in steatotic liver but not in the liver of BDL-mice when compared to controls. Adiponectin mRNA was not detected in human liver samples or primary human hepatocytes nor in rat liver but recombinant adiponectin is taken up by isolated hepatocytes *in-vitro*. AdipoR1 mRNA and AdipoR1 protein levels are similar in the liver tissue of control and fat fed animals whereas AdipoR2 mRNA is induced. AdipoR2 mRNA and AdipoR1 mRNA and protein is suppressed in the liver of BDL-mice.

CONCLUSION: Our studies show reduced circulating adiponectin in a rat model of fatty liver disease whereas circulating adiponectin is elevated in a mouse model of cirrhosis and similar findings have been described in humans. Diminished hepatic expression of adiponectin

INTRODUCTION

Obesity and especially visceral fat accumulation cause insulin resistance, a common risk factor for hepatic steatosis. Fatty liver is thought to represent the first step towards the subsequent development of liver fibrosis. Impaired mitochondrial function provides the second hit and promotes the generation of reactive oxygen species, which promote lipid peroxidation, the release of inflammatory cytokines, death of hepatocytes and activation of hepatic stellate cells. Non-alcoholic steatohepatitis (NASH) is a progressive disorder that can lead to liver cirrhosis and even hepatocellular carcinoma^[1].

Adiponectin is highly abundant in human serum and is secreted by adipose tissue in inverse proportion to the body mass index^[2]. Adiponectin improves whole body insulin sensitivity and in addition exerts anti-inflammatory effects by reducing NF κ B activation^[3]. Low adiponectin levels are associated with NASH independent of insulin resistance and body mass index indicating a protective effect for adiponectin in liver disease^[4]. This idea is supported by studies in rodents where recombinant adiponectin given to leptin-deficient ob/ob mice ameliorates hepatic steatosis and normalizes alanine aminotransferase levels^[5]. Besides these protective effects on fatty liver disease adiponectin attenuates T-cell mediated hepatic inflammation by reducing the release of proinflammatory cytokines, the activation of hepatic stellate cells and cell death of hepatocytes^[6].

Two 7-transmembrane proteins, AdipoR1 and AdipoR2, have been identified to function as adiponectin receptors^[7]. AdipoR1 mRNA is mainly expressed in the

human heart and skeletal muscle, whereas AdipoR2 was supposed to be the main receptor in the liver^[7]. Recently a prominent protein expression of AdipoR1 in primary hepatocytes was demonstrated indicating that AdipoR1 may also be important in hepatic signal transduction^[8]. Adiponectin activates the AMP-activated protein kinase (AMPK) and PPAR α ^[7] but may also inhibit the binding of growth factors to their corresponding receptors independent of AdipoR1 and AdipoR2^[9].

Although there is a well documented relationship between low adiponectin and liver disease, the role of adiponectin receptors is less clear^[4,6]. In addition, mainly AdipoR2 has been analysed with regard to liver function^[10,11]. Therefore the expression of AdipoR1 was investigated in rodent models of fatty liver disease and liver cirrhosis.

MATERIALS AND METHODS

Subjects

Wistar rats were fed a standard rodent chow (6 animals) or a high fat diet for twelve weeks (six animals) as recently described^[12]. Male C57Bl/6J mice (body weight 25-30 g) underwent common bile duct ligation (BDL) and transection as previously reported^[13]. Another group of animals was sham-operated to serve as a control. For analysis of adiponectin or AdipoR1 protein four control and five BDL mice were used. Analysis of mRNA expression was performed with total RNA isolated from the liver of eight control and four BDL animals. All animal procedures were performed under the guidelines set by The University of Regensburg Institutional Animal Care and Use Committee. Primary hepatocytes from three different donors were isolated and cultivated as described before^[14]. Tissue samples from human liver resection were obtained from patients undergoing partial hepatectomy. Experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR (Human Tissue and Cell Research), with the informed patient's consent^[15] approved by the local ethical committee of the University of Regensburg.

Culture media and reagents

RPMI medium was from Biochrom (Southborough, MA, USA), RNeasy Mini Kit from Qiagen (Hilden, Germany) and oligonucleotides were synthesized by Metabion (Planegg-Martinsried, Germany). LightCycler FastStart DNA Master SYBR Green I was purchased from Roche (Mannheim, Germany). AdipoR1 peptide antibody was raised as recently described^[8]. AdipoR2 protein was not analysed because several antibodies investigated did not specifically detect in-vitro translated AdipoR2 by immunoblot (own unpublished results).

ELISAs for human and mouse adiponectin and human recombinant adiponectin were from R&D systems (Wiesbaden-Nordenstadt, Germany). ELISAs for rat adiponectin from BioCat (Heidelberg, Germany). PI3-Kinase p85 antibody was from Upstate (Lake Placid, NY, USA). Recombinant human leptin was from Sigma Chemical (Deisenhofen, Germany) and 100 μ g/L were used.

Monitoring of gene expression by real-time RT-PCR

Real-time RT-PCR was performed as recently described^[16].

Table 1 Oligonucleotides used for real-time RT-PCR

Name	Sequence
AdipoR1 uni (h)	5'-GGGGAATTCTCTCCACAAAGGATCTGTG GTG-3'
AdipoR1 rev (h)	5'-GGGCTGCAGTTAAGTTTCTGTATGAATGCG GAAGAT-3'
AdipoR2 uni (h, m)	5'-GGGGAATTCAACGAGCCAACAGAAAACCG ATTG-3'
AdipoR2 rev (h, m)	5'-GGGCTGCAGCTAAATGTTGCCTGTTTCTGTG TGTAT-3'
β -actin uni (h)	5'-CTACGTCGCCCTGGACTTCGAGC-3'
β -actin rev (h)	5'-GATGGAGCCGCCGATCCACACGG-3'
AdipoR1 uni (m)	5'-AGGCCTGTCCACCATCAC-3'
AdipoR1 rev (m)	5'-CAGAAGGAGCCCCATTGC-3'
AdipoR1 uni (r)	5'-CGACAGGCCTAAGTGTCAT-3'
AdipoR1 rev (r)	5'-CTTACCCTTCTCTCCAGCA-3'
AdipoR2 uni (r)	5'-GAAGGAGGGTCAACTCACCA-3'
AdipoR2 rev (r)	5'-CATCAAGTTGGTGCCTTTT-3'
β -actin uni (m)	5'-TGGAATCTGTGGCATCCATG-3'
β -actin rev (m)	5'-TAAAACGCAGCTCAGTAACAG-3'
adiponectin (h)	5'-CATGACCAGGAAACCACGACT-3'
adiponectin (h)	5'-TGAATGCTGAGCGGTAT-3'

h: Human; m: Mouse; r: Rat.

The primers are listed in Table 1. Amplification in the LightCycler capillaries was done for 45 cycles with initial incubation of ten minutes at 95°C for activation of Taq polymerase. Cycling parameters were 15 s at 95°C, ten seconds 60°C and ten seconds at 72°C. The second derivative method was used for quantification with the LightCycler software. For quantification of the results the standard curve method was used. Normalization was performed by dividing each value calculated for a specific gene by the value of the corresponding housekeeping gene. QuantiTect Primer Assays (Qiagen) for use in real-time RT-PCR with SYBR Green detection were used to determine rat adiponectin mRNA.

SDS-PAGE and immunoblotting

The cells or tissues were solubilized in RIPA buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis and were transferred to PVDF membranes (Bio-Rad, Germany). Incubations with antibodies were performed in 1% BSA in PBS, 0.1% Tween overnight. Detection of the immune complexes was carried out with the ECL Western blot detection system (Amersham Pharmacia, Deisenhofen, Germany).

Statistical analysis

Data are represented as Box Plots (Sigma Plot) indicating the median, the upper and lower quartile, the largest and the lowest value in the data set. Statistical differences were analysed by Student's *t*-test (MS Excel) and a value of *P* < 0.05 was regarded as statistically significant.

RESULTS

Expression of AdipoR1 was analysed in HepG2 cells treated with insulin or leptin. HepG2 cells were treated

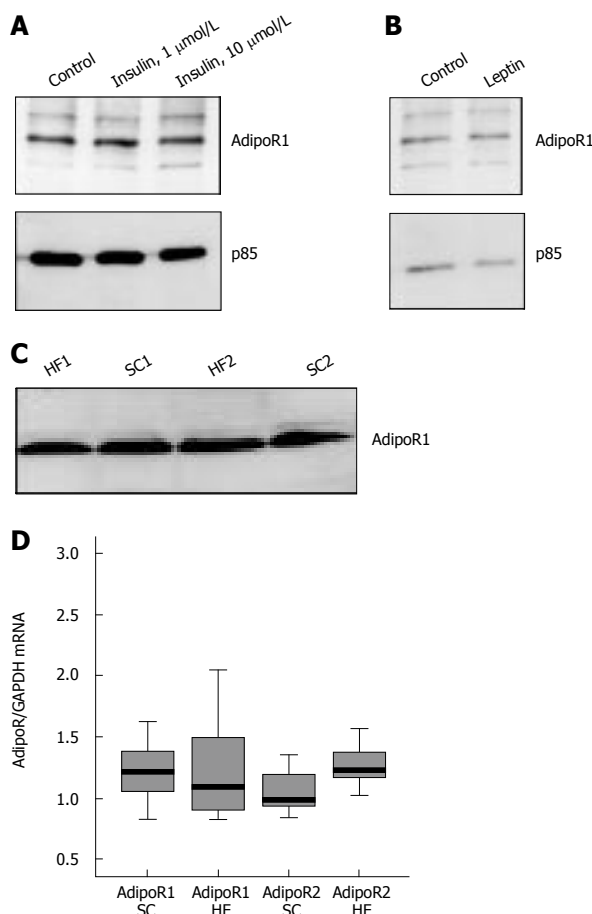


Figure 1 AdipoR1 mRNA and protein and AdipoR2 mRNA in fatty liver disease. **A:** Expression of AdipoR1 in HepG2 incubated with 1 $\mu\text{mol/L}$ or 10 $\mu\text{mol/L}$ insulin for 4 h; **B:** Analysis of AdipoR1 in HepG2 treated with recombinant leptin for 24 h; **C:** AdipoR1 abundance in the liver of rats kept on a standard chow (SC) or a high fat diet (HF); **D:** AdipoR1 and AdipoR2 mRNA expression in the liver of rats kept on a standard chow (SC) or a high fat diet (HF). For normalization GAPDH mRNA levels were determined.

with insulin, 1 $\mu\text{mol/L}$ and 10 $\mu\text{mol/L}$ for four hours (Figure 1A) and six hours (not shown) or leptin for 24 h. AdipoR1 was analyzed by immunoblot and was found not to be regulated by insulin or leptin (Figures 1A and B). AdipoR1 and AdipoR2 mRNA expression was determined in insulin treated cells and was similar in controls and insulin-incubated HepG2 cells (not shown).

AdipoR1 protein and mRNA as well as AdipoR2 mRNA were analysed in the liver of rats on a standard chow (SC) or on a high fat (HF) diet. AdipoR1 protein level is similar in these animals (Figure 1C) and real-time RT-PCR revealed no alterations in the mRNA expression of AdipoR1 whereas AdipoR2 mRNA is induced with a relative expression of 1.0 ± 0.2 in SC and 1.3 ± 0.2 in the HF group ($P = 0.004$) (Figure 1D). RT-PCR data were normalized by the corresponding GAPDH values which were not different between the two groups.

Systemic and liver adiponectin were measured by ELISA. Circulating adiponectin is significantly reduced in fat-fed animals with 4.9 ± 1.1 mg/L adiponectin compared to 6.2 ± 8 mg/L in SC fed rats ($P = 0.02$) (not shown). Liver adiponectin is also lower in fat rats with 8.5 ± 2.4 ng adiponectin in 1 μg liver tissue when compared to controls with 11.9 ± 1.9 ng adiponectin ($P = 0.01$) (Figure 2A).

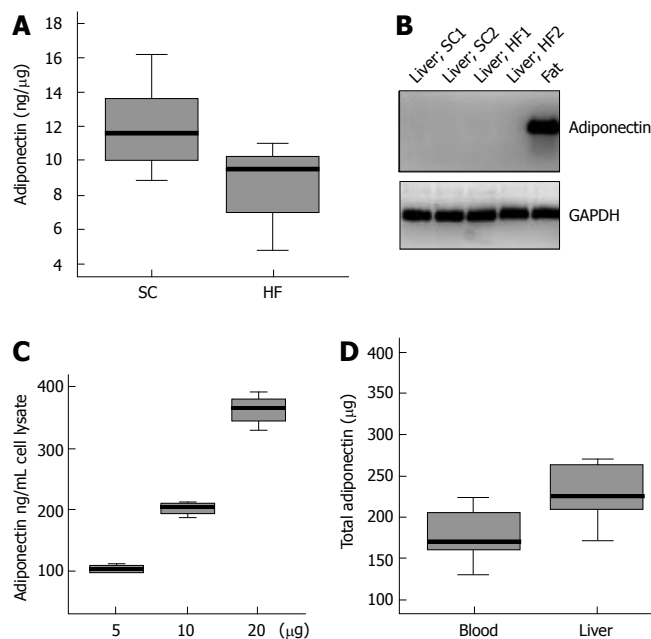


Figure 2 Hepatic adiponectin protein and mRNA in fatty liver disease. **A:** Adiponectin in the liver of standard chow (SC) and high fat diet (HF) animals. Concentration of adiponectin is ng per μg liver tissue; **B:** Adiponectin mRNA in the liver of rats kept on a standard chow (SC) or a high fat diet (HF). Fat tissue was analysed as a positive control; **C:** Primary hepatocytes were incubated with 5, 10 or 20 mg/L adiponectin for 24 h and adiponectin was determined in the cell lysates; **D:** Total systemic and hepatic adiponectin was calculated.

Adiponectin in the liver may be derived from the circulation or originate from liver cells. Adiponectin mRNA was analysed by RT-PCR with total RNA isolated from rat liver of HF and SC animals and rat adipose tissue as a positive control. Adiponectin mRNA was not detected in any RNA isolated from total liver by RT-PCR and 45 cycles of amplification but was easily amplified from adipose tissue RNA (Figure 2B). In addition, adiponectin mRNA was not detected in human liver, in isolated primary human hepatocytes or in HepG2 cells (not shown). Therefore hepatic adiponectin is most likely taken up by liver cells.

To test this hypothesis primary human hepatocytes were incubated with 5, 10 and 20 mg/L recombinant adiponectin for 24 h or PBS as solvent control in serum-free medium. Whereas adiponectin was not detected in the control cells and therefore is below 40 ng/L, adiponectin was found in the cell lysates of hepatocytes incubated with increasing amounts of recombinant protein and was 111 ± 3 $\mu\text{g/L}$, 200 ± 18 $\mu\text{g/L}$ and 360 ± 43 $\mu\text{g/L}$, respectively (Figure 2C). This indicates that about 0.02% of the extracellular adiponectin is taken up by hepatocytes and the uptake correlates to the adiponectin concentration in the medium.

Total circulating adiponectin in rats was calculated and the blood volume was estimated as described (Blood volume (mL) = $0.06 \times \text{body weight} + 0.77$)^[17]. Total liver adiponectin was also calculated by multiplication of hepatic adiponectin concentration with the corresponding weight of the liver. Adiponectin in the blood was 178 ± 31 μg and in the liver 241 ± 59 μg indicating that total liver adiponectin is higher than circulating adiponectin ($P = 0.03$) (Figure 2D). However, this is only a rough estimate because the blood volume of the liver was not included

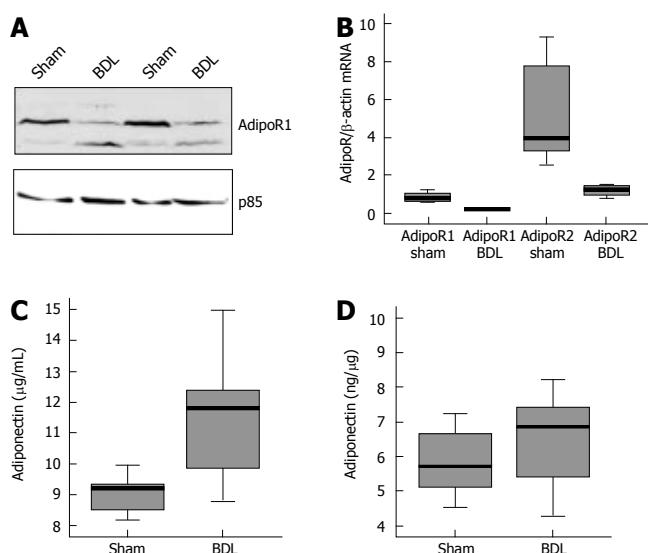


Figure 3 AdipoR1 and adiponectin in liver cirrhosis. **A:** AdipoR1 abundance in the liver of sham-operated or BDL mice; **B:** AdipoR1 and AdipoR2 mRNA expression in the liver of sham-operated or BDL mice. For normalization β -actin levels were determined; **C:** Systemic adiponectin in control and cholestatic animals; **D:** Adiponectin in the liver of sham-operated or BDL mice. Concentration of adiponectin is mg per g liver tissue.

in the calculation. Furthermore, adiponectin may be not equally distributed in the whole liver and levels may vary between different biopsies taken from the same organ.

AdipoR1 and AdipoR2 were also determined in a rodent model of liver cirrhosis. AdipoR1 protein was analysed in the liver of control mice and animals after BDL by immunoblot and was found reduced in all BDL animals investigated (Figure 3A). AdipoR1 and AdipoR2 mRNA were determined by real-time RT-PCR in control and BDL mice. Relative abundance of AdipoR1 mRNA was 0.8 ± 0.3 in sham and 0.2 ± 0.1 in BDL mice ($P = 0.0004$), AdipoR2 mRNA was 5.2 ± 2.6 in controls and 1.2 ± 0.3 in BDL animals ($P = 0.007$) (Figure 3B). Normalization was performed using β -actin as housekeeping gene which was similarly expressed in both groups ($P = 0.14$). Systemic adiponectin was significantly higher in the BDL group with 9.0 ± 0.6 mg/L in the control and 11.1 ± 2.0 mg/L in the BDL animals ($P = 0.03$) (Figure 3C) whereas hepatic adiponectin was similar with 5.8 ± 1.0 mg/g in sham and 6.4 ± 1.5 mg/g in the BDL mice ($P = 0.4$) (Figure 3D).

To identify mediators that are responsible for the suppression of AdipoR1 in liver cirrhosis, primary hepatocytes and HepG2 cells were incubated for 24 h with either LPS (1 and 10 mg/L), TNF (10 μ g/L), supernatants of activated hepatic stellate cells, CCl₄ (1 mmol/L and 3 mmol/L) or actinomycin D (10 mg/L). AdipoR1 protein was not found reduced in cells treated with these mediators (not shown).

DISCUSSION

Although AdipoR2 has been suggested to represent the main adiponectin receptor in the liver we recently demonstrated significant expression of AdipoR1 mRNA and protein in primary human hepatocytes and HepG2 cells^[8]. In the current study AdipoR1 mRNA and protein,

AdipoR2 mRNA and abundance of circulating and hepatic adiponectin in rodent models of steatotic and cirrhotic liver was investigated.

Rats fed a HF diet develop insulin resistance and fatty liver disease^[12]. AdipoR1 mRNA and protein is similar when total liver tissue isolated from rats on a standard diet or HF animals was analyzed. Furthermore, insulin did not alter AdipoR1 mRNA or protein in HepG2 cell. Leptin deficient ob/ob mice are obese and insulin resistant and hepatic AdipoR1 mRNA was found similarly expressed in these animals when compared to controls^[18]. These data indicate that reduced insulin sensitivity is not associated with reduced AdipoR1 expression in the liver.

AdipoR2 mRNA is about 25% higher in the fatty liver of HF rats whereas in the ob/ob mice AdipoR2 mRNA is not altered^[18]. AdipoR2 mRNA was also investigated in humans and is significantly elevated in fatty liver disease when compared to normal liver^[19] in one study whereas a second study could not detect alterations in AdipoR2 mRNA^[10,20]. However, protein expression has not been investigated so far but is important to clarify abundance of hepatic AdipoR2 in the metabolic syndrome.

Systemic adiponectin is significantly lower in the HF animals and reflects the human situation where circulating adiponectin has an inverse proportion to the body mass index^[2]. In accordance with the lower systemic adiponectin levels, hepatic adiponectin is also reduced in the fat rat. Adiponectin mRNA was not detected in rat liver indicating that hepatic adiponectin may be taken up by the cells. Adiponectin was not found in primary human hepatocytes cultivated *in vitro* for three days but incubation of these cells with 5, 10 or 20 mg/L recombinant adiponectin for 24 h elevated cellular adiponectin in a concentration dependent way. This may indicate that adiponectin is taken up by hepatocytes either from the circulation or the portal vein.

The common and final state of all chronic liver diseases is liver cirrhosis. A well known model of extrahepatic biliary obstruction is common bile duct ligation in mice^[21]. AdipoR1 mRNA and protein and AdipoR2 mRNA were found reduced in the cirrhotic liver of BDL animals. Systemic adiponectin is elevated in animals with liver cirrhosis, a phenomenon also described in a study investigating mice and humans^[22]. However, hepatic adiponectin was similar in sham and BDL mice. It was suggested that adiponectin is excreted via the bile and an impaired liver function in cirrhosis may reduce biliary loss of circulating adiponectin explaining elevated systemic levels^[22]. However, this hypothesis presumes reduced hepatic adiponectin concentrations when compared to control animals and therefore is not supported by our findings.

Although adiponectin was detected in the liver of mice and rats, adiponectin mRNA could not be amplified in rat liver, human liver or isolated human hepatocytes indicating that adiponectin is not produced by hepatocytes or other cells of the liver tissue. These results are in agreement with several studies^[10,20] but contradict the results of other reports^[11,22] and there is currently no explanation for these different findings. However, hepatocytes take up significant amounts of adiponectin and this may explain at least in

part why adiponectin is detected in the liver.

One limitation of the current study is that only four to eight animals could be analyzed per experiment and the investigations would benefit from a higher number of rodents. Nevertheless low circulating adiponectin in obesity^[2] and high levels in liver cirrhosis^[22] have also been reported in human studies and are in accordance with our findings.

All in all, our experiments show reduced circulating adiponectin in a rodent model of fatty liver disease and elevated adiponectin and diminished expression of adiponectin receptors in BDL-induced liver cirrhosis in mice.

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C-reactive protein is a prognostic indicator in patients with perihilar cholangiocarcinoma

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Abstract

AIM: To evaluate prognostic indicators for the outcome of patients with perihilar extrahepatic cholangiocarcinoma in an unselected cohort.

METHODS: We retrospectively analyzed 98 patients with perihilar cholangiocarcinoma. Twenty-three patients (23.5%) underwent tumor resection. Patients with non-resectable tumors underwent either transpapillary or percutaneous transhepatic biliary drainage. Additionally, 32 patients (32.7%) received photodynamic therapy (PDT) and 18 patients (18.4%) systemic chemotherapy. Predefined variables at the time of diagnosis and characteristics considering the mode of treatment were entered into a Cox's proportional hazards model. Included in the analysis were age, tumor stage following the modified Bismuth-Corlette classification, bilirubin, prothrombin time (PT), C-reactive protein (CRP), carbohydrate antigen 19-9 (CA19-9), history of weight loss, surgical resection, chemotherapy and PDT.

RESULTS: The Kaplan-Meier estimate of overall median survival was 10.5 (95%CI: 8.4-12.6) mo. In the univariate analysis, low Bismuth stage, low CRP and surgical resection correlated significantly with better survival. In the multivariate analysis, only CRP ($P = 0.005$) and surgical resection ($P = 0.029$) were found to be independently predictive of survival in the cohort. Receiver operating characteristic (ROC) analysis identified a CRP level of 11.75 mg/L as the value associated with the highest sensitivity and specificity predicting a survival > 5 mo. Applying Kaplan-Meier analysis, patients with a CRP < 12 mg/L at the time of diagnosis had a significantly longer median survival than patients with higher values (16.2 vs 7.6 mo; $P = 0.009$).

CONCLUSION: This retrospective analysis identified CRP level at the time of diagnosis as a novel indicator for the prognosis of patients with perihilar cholangiocarcinoma. It should be evaluated in future prospective trials on this entity.

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Key words: Perihilar cholangiocarcinoma; Prognostic factors; C-reactive protein; Resection; Outcome

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INTRODUCTION

Cholangiocarcinoma is a rare tumor^[1]. The global incidence varies between 0.5 and 1.1 per 100 000^[2]. High-risk groups have been defined. Thus, the life-time risk of intrahepatic and extrahepatic cholangiocarcinoma among patients with primary sclerosing cholangitis (PSC) ranges between 8%-20%^[3]. Further risk factors for the occurrence of cholangiocarcinoma are infections with liver flukes^[4], hepatolithiasis^[5], choledochal cysts^[6] or application of thorotrast^[7]. While recent data show that incidence and mortality rates of intrahepatic cholangiocarcinoma are increasing in several areas in the world, the incidence and mortality rates of extrahepatic carcinoma are declining^[8]. The 5-year survival of patients with extrahepatic cholangiocarcinoma is poor and was found to be less than 20% in a large population-based epidemiological study from the United States^[1].

Perihilar cholangiocarcinoma is mostly diagnosed at an advanced stage. Therefore, more than two-thirds of patients are not suitable for surgery due to either expansion of the tumor or age and comorbidity^[9]. Nevertheless, the prognosis of patients undergoing tumor resection has improved in recent years owing to advancements in surgical techniques resulting in a more aggressive resectional approach^[10,11]. Furthermore, liver transplantation may be an option in highly selected patients after neo-adjuvant

radiochemotherapy and invasive staging^[12,13].

Prognostic factors predicting the outcome of patients undergoing tumor resection have recently been extensively evaluated^[10,14]. In contrast, less attention has been paid to overall outcome and possible prognostic indicators in unselected patients suffering from cholangiocarcinoma. Therefore, we performed a retrospective analysis of 98 consecutive patients with perihilar cholangiocarcinoma treated at a tertiary medical center within a period of 5 years in order to identify the most relevant predictors of outcome.

MATERIALS AND METHODS

Data acquisition

Using our hospital database, we identified the records of 98 consecutive unselected patients with extrahepatic perihilar cholangiocarcinoma type Bismuth I to IV admitted to our hospital between October 1997 and March 2003. Charts were reviewed retrospectively. Data for further analysis were available from all patients.

Diagnostic criteria

Cholangiography showed a perihilar stricture in all patients. Positive histology and/or cytology were present in 68 (69.4%) patients. In the remaining patients, diagnosis was made by the coexistence of a CA19-9 serum level greater than 250 IU/L and typical findings at cholangiography, ultrasound and CT scan.

We recorded patients' age, gender, clinical presentation, tumor stage following the modified Bismuth-Corlette classification^[9], laboratory parameters at presentation (blood count, CRP, bilirubin, alkaline phosphatase, GPT, CA19-9), histology, cytology, type of medical treatment and outcome including date of death.

Statistical analysis

Numeric data were recorded as median and range or 95% confidence intervals (95% CI). To identify prognostic factors, we used the Cox's proportional hazards regression analysis. Survival analysis was performed using the Kaplan-Meier method and comparisons were made employing the log rank test. The Mann-Whitney rank sum test was used for inter-group comparisons. Statistical analysis was performed using the SPSS®- (SPSS Inc., Chicago, IL, USA) and the StatView 5.0®-Software (Version for Windows; SAS Institute Inc., Cary, NC, USA). A *P* value less than 0.05 was considered statistically significant.

RESULTS

Demographics and results of initial evaluation

We conducted this retrospective analysis on 98 consecutive patients (female/male: 48/50) with a median age of 69.5 (range: 35.8-89.9) years. Two patients of the cohort were known to suffer from PSC. Major clinical symptoms at admission were jaundice (73.5%), weight loss (43.9%) and pruritus (33.7%), whereas pain (22.5%), ascites (11.2%) and fever (9.2%) were present in less than one third of patients. The tumors were described as Bismuth types I

Table 1 Laboratory findings at time of diagnosis

Value (normal range)	Median	Range
Total bilirubin (0.1-1.2 mg/L)	9.1	0.3 38.3
Alkaline phosphatase (50-175 U/L)	375	26 2572
ALT (GPT) (- 19 U/L)	61.5	8 464
Leucocytes (4.3-10.5 G/L)	11	3.6 95
CRP (- 3 mg/L)	8.4	0.06 207
CA19-9 (0.25-20 U/L)	232.9	0.25 24385
PT (70%-130%)	98.5	36 190

ALT: Alanin-Amino-Transferase; GPT: Glutamat-Pyruvat-Transaminase; CRP: C-reactive protein; CA 19-9: Carbohydrate-Antigen 19-9; PT: Prothrombin time.

(*n* = 12), II (*n* = 7), III (*n* = 30) and IV (*n* = 49), respectively. The laboratory findings at time of diagnosis are given in Table 1. CA19-9 levels did not correlate significantly to either serum bilirubin level (*r* = 0.068; *P* = 0.54) or Bismuth stage (*r* = 0.085; *P* = 0.44). Higher CRP-levels correlated significantly to leukocyte count (*r* = 0.569; *P* < 0.0001), but did not depend on bilirubin levels (*r* = 0.153; *P* = 0.16) and tumor extent according to the Bismuth-Corlette classification (*r* = 0.160; *P* = 0.15).

Modality of treatment

Explorative laparotomy was performed in 43 patients (43.9%), and tumor resection could be performed in 23 (23.5%) patients of the cohort. Surgical therapy consisted of resection of the extrahepatic bile-ducts in 9 (39.1%) patients, partial duodenopancreatectomy with hilar resection in 2 (8.7%) patients, hilar resection with right hemihepatectomy in 5 (21.7%) patients, hilar resection with left hemihepatectomy in 4 (17.4%) patients, hilar resection with atypical liver resection in 2 (8.7%) patients and hepatectomy with consecutive liver transplantation in 1 (4.3%) patient. The resected patients were younger, had a lower Bismuth stage and had lower levels of serum bilirubin at diagnosis than the patients who did not undergo surgery, whereas CRP at diagnosis did not differ significantly between both groups (Table 2).

Patients with non-resectable tumors underwent either transpapillary or percutaneous transhepatic biliary drainage. Sixty-two (82.6%) of these patients received unilateral or bilateral plastic stents as biliary endoprosthesis, whereas in 34 (45.3%) patients, metal stents were placed during the course of the disease. In 30 (40%) patients, a percutaneous drainage had to be placed on at least one occasion during their clinical course.

Fifty-one patients received additional therapy. This therapy consisted of intraluminal photodynamic therapy using porfimer sodium (PhotofrinTM, Axcan, Canada) in 32 patients and systemic chemotherapy in 18 patients.

Survival analysis

At the end of observation, 85 of 98 (86.7%) patients had deceased with a median survival of 8.8 (0.8-55.1) mo. Sixteen patients were alive with a median follow-up of 12.3 (1.4-71.7) mo. The Kaplan-Meier estimated overall median survival was 10.5 (95% CI: 8.4-12.6) mo (Figure 1).

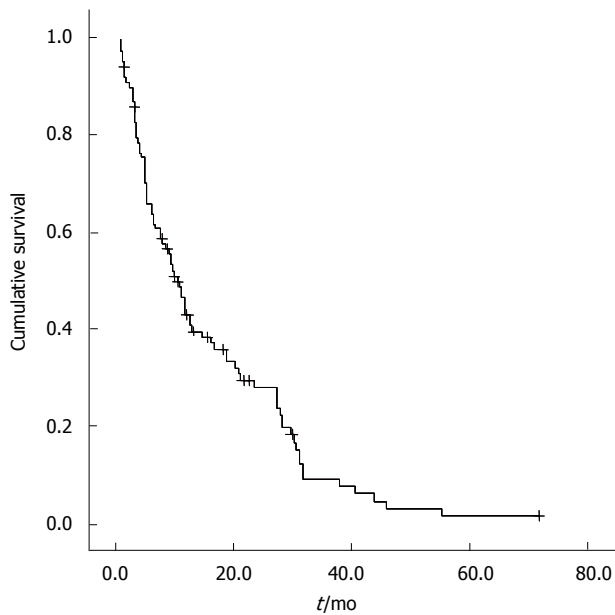


Figure 1 Kaplan-Meier estimate for survival of the whole cohort.

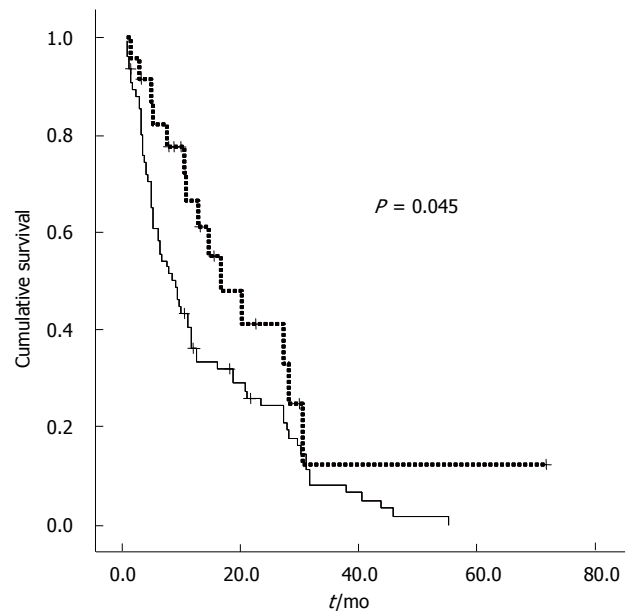


Figure 3 Kaplan-Meier estimate for survival of patients, who underwent resection (dotted line) versus non-resected patients (solid line).

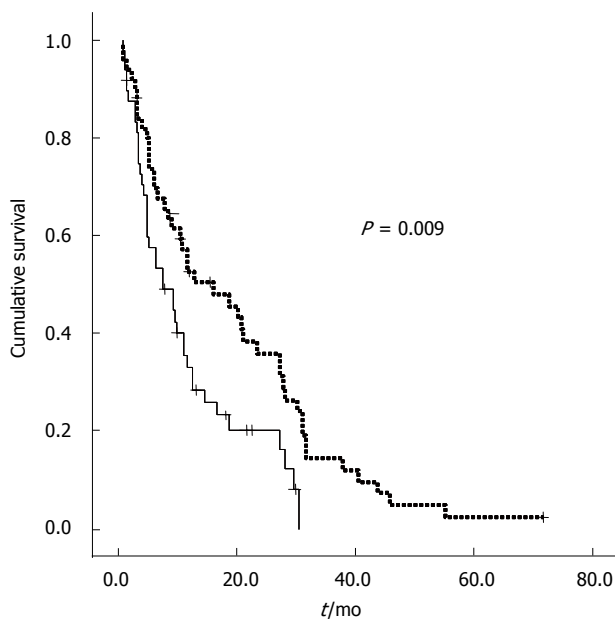


Figure 2 Kaplan-Meier estimate for patients with a serum CRP level < 12 mg/L (dotted line) versus ≥ 12 mg/L (solid line).

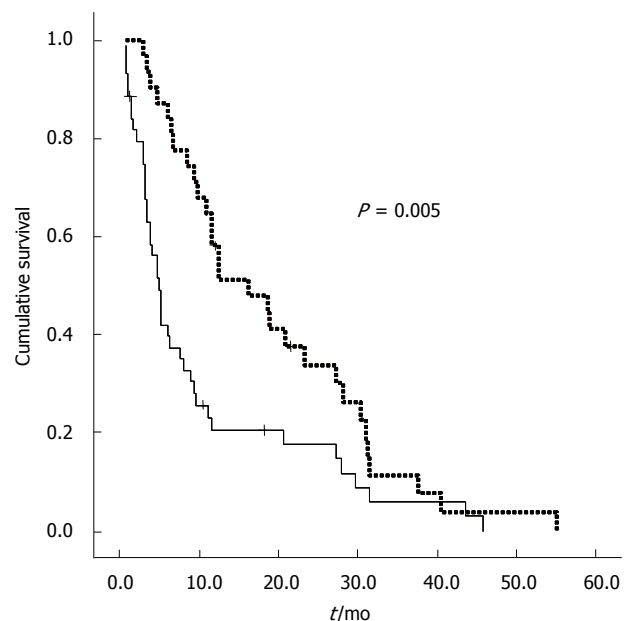


Figure 4 Subgroup analysis of patients without surgical resection: Kaplan-Meier estimate for survival of patients treated with PDT (dotted line) versus non-treated patients (solid line).

Prognostic factors and impact of treatment modality

The parameters examined and the results of the uni- and multivariate analyses are shown in Table 3. In the univariate analysis, low Bismuth stage, low CRP and surgical resection correlated significantly with better survival. In the multivariate analysis, only surgical resection ($P = 0.029$) and CRP ($P = 0.005$) were found to be independently predictive of survival in the cohort. ROC analysis identified a CRP level of 11.75 mg/L as the value associated with the highest sensitivity and specificity to identify patients surviving more than 5 mo.

Patients with a CRP level < 12 mg/L at the time of

diagnosis had a significantly longer median estimated survival than patients with higher CRP values (16.2 *vs* 7.6 mo; $P = 0.009$) (Figure 2). The median survival in the subgroup of patients who underwent resection was significantly longer compared to patients receiving palliative treatment [16.6 (95% CI: 7.7-25.5) *vs* 9.0 (95% CI: 5.6-12.5); $P = 0.045$] (Figure 3). In contrast to the analysis of the whole cohort, in the subgroup of patients with irresectable tumors, PDT was associated with a significant improvement of survival [16.2 (95% CI: 7.0-25.5) *vs* 5.0 (95% CI: 3.8-6.3) mo ($P = 0.005$)] (Figure 4). Systemic chemotherapy was not correlated to a better outcome

Table 2 Inter-group comparison between patients undergoing surgical resection and patients with irresectable tumors

	Resection (<i>n</i> = 23)	No resection (<i>n</i> = 75)	<i>P</i>
Age	65.8 (36.4-74.1)	71.8 (35.8-90)	0.0003
Bismuth	II (I-IV)	IV (I-IV)	0.001
Bilirubin	3.3 (0.3-17.5)	9.6 (0.4-38.3)	0.0026
CRP	15.6 (0.5-101)	9.9 (0.1-207)	0.445
CA19-9	71 (0.3-2910)	322 (0.3-24 385)	0.0098

Data are expressed as median (range).

neither in the multivariate analysis of the whole group nor in the subgroup of non-resected patients [11.6 (95% CI: 0.6-25) with chemotherapy *vs* 8.6 (95% CI: 5.0-12.2) mo without chemotherapy; *P* = 0.33].

DISCUSSION

Our study evaluated outcome and prognostic factors in a large series of unselected patients with perihilar cholangiocarcinoma treated at a tertiary medical center. The prognosis of these patients was poor. The median overall survival in our series was only 10.5 mo. Serum CRP level at diagnosis was identified as a new prognostic indicator for patients with perihilar cholangiocarcinoma. Surgical resection was also associated with prolonged survival. Moreover, in the subgroup of patients with irresectable tumors, additional therapy with PDT apart from biliary drainage, but not chemotherapy, was correlated with a better outcome. Certainly, particularly our data on the impact of treatment modalities on survival are influenced by all the restrictions of a retrospective analysis. There may be biases, such as selection for surgery and less complete follow-up in comparison to a prospective study. Unfortunately, prospective data on the clinical course of non-selected patients with perihilar cholangiocarcinoma are rare. Nevertheless, we were able to analyze a relatively large unselected cohort.

Prognostic factors in patients with cholangiocarcinoma undergoing resection have been extensively evaluated in retrospective series^[11]. In a large series presented by Jarnagin *et al*^[15], negative histologic margins, concomitant partial hepatectomy and a well-differentiated tumor were associated with an improved outcome. Accordingly, residual tumor as well as lymph node involvement were significant prognostic factors in a cohort of long-term-survivors^[14]. Much less is known about the overall outcome of a more heterogeneous non-selected cohort with respect to its possible prognostic factors. Weight loss has previously been reported to be significantly associated with the outcome of patients with malignant strictures of the distal bile duct^[16]. However, this factor could not be confirmed in our cohort of patients with perihilar tumors. Although a retrospective study of 49 cases of resected hilar cholangiocarcinoma identified total bilirubin greater than 10 mg/L to be associated with poorer survival^[17], the bilirubin level was not significantly correlated to the outcome in our study. CRP, on the other hand, was a statistically significant prognostic factor, even in the multivariate analysis. Patients with a CRP <

Table 3 Results of univariate analysis for prognostic factors of survival

Variables	Hazard ratio	90% CI	<i>P</i>
Age	1.028	1.004 1.052	0.053
Bismuth type	0.784	0.656 0.927	0.023
Bilirubin	1.024	1.002 1.046	0.067
PT	0.996	0.989 1.004	0.402
CRP ^a	1.007	1.003 1.011	0.002
CA19-9	1.000	1.000 1.000	0.079
History of weight loss (yes/no)	1.125	0.783 1.615	0.592
Resection (yes/no) ^a	0.559	0.345 0.908	0.049
Chemotherapy (yes/no)	0.700	0.437 1.123	0.215
PDT (yes/no)	0.670	0.458 0.980	0.084

PT: Prothrombin time, CRP: C-reactive protein, CA 19-9: Carbohydrate-Antigen 19-9. Significant in the multivariate analysis (^a*P* < 0.05).

12 mg/L at the time of diagnosis had a significantly longer median survival than patients with higher CRP values (16.2 *vs* 7.6 mo; *P* = 0.009). CRP belongs to the family of acute-phase proteins. Its concentration changes in response to injury, infection and neoplasia. It is up-regulated by cytokines, such as interleukin-8 (IL-8), interleukin-6 (IL-6) and tumor necrosis factor α (TNF- α)^[18]. *In vitro* studies have identified IL-6 to be an autocrine growth factor of cholangiocarcinoma (CC) cell lines^[19,20], in which it induces the expression of the anti-apoptotic protein Mcl-1^[21]. Moreover, IL-6 was found to be markedly elevated in the serum of patients with CC and dropped sharply after resection^[22]. Thus, high CRP levels might reflect an increased IL-6 level in patients with advanced cholangiocarcinoma. In this respect, the lack of IL-6 serum level determination displays a limitation of our study. In general, increased CRP levels in malignant disease could also be caused by an inflammatory response to tumor invasion^[23]. Others showed in immunohistochemical studies that neoplastic tissue itself can express CRP^[24]. In cholangiocarcinoma, one might also speculate that elevated CRP serum levels were caused by complicated tumor-induced strictures and subsequent cholangitis. Whereas in our study initial CRP levels correlated to leukocyte count, they were not significantly correlated to tumor size as assessed by the Bismuth-Corlette classification. Interestingly, increased serum CRP levels also correlated with shorter survival in patients with other gastrointestinal malignancies, including pancreatic, esophageal and colorectal cancer^[25-27]. Recently, a CRP level \leq 1.0 mg/dL was identified as favorable prognostic factor in a group of 65 patients with biliary tract cancers receiving chemotherapy^[28]. However, this cohort consisted of 82% patients with gallbladder carcinoma, an entity with potentially different biological behavior and less frequent occurrence of cholestasis as compared to ours.

CA19-9 has been shown to be useful in the diagnostic evaluation of cholangiocarcinoma^[29,30] and the resectability of intrahepatic and periampullary carcinomas^[31,32]. Those of our patients who underwent resection had significantly lower CA19-9 levels at diagnosis, which might reflect a smaller tumor mass, but yet the marker was not correlated to overall outcome. This is in contrast to patients with

inoperable pancreatic cancer undergoing chemotherapy with gemcitabine, in whom CA 19-9 was prognostic^[33].

The definitive role of chemotherapy and radiotherapy in the treatment of CC has not been fully established, although both options are commonly used^[34]. In our cohort, a small number of patients receiving chemotherapy did not show favorable outcome compared to those without. Also PDT, which had been shown to be a promising palliative approach in several non-randomized and randomized studies on patients with irresectable cholangiocarcinoma, failed to be associated with favorable outcome in the overall analysis. However, it demonstrated a significant influence on survival in the subgroup of non-resected patients. Survival in these patients is comparable to previously published results from prospective trials^[35-37].

In accordance with the literature, somewhat one fourth of our patients (24.8%) underwent surgical resection. In the univariate and the multivariate analyses, resection was significantly associated with a better outcome. Patients undergoing resection of their tumor were significantly younger, although age itself was not an independent prognostic parameter. Conclusions of the influence of tumor resection on the outcome of patients with perihilar CC in comparison to conservative treatment are clearly limited by the retrospective character of this analysis, which implements possible bias by patient selection.

In summary, our study evaluated the outcome of a heterogeneous non-selected cohort of patients with cholangiocarcinoma. In agreement with previous studies, surgical resection was identified as a prognostic factor for prolonged survival. In addition, the serum level of CRP at diagnosis was identified as a novel and independent prognostic indicator in patients suffering from perihilar cholangiocarcinoma and should, therefore, be considered as a prognostic parameter in the design of future prospective studies on this kind of patients.

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Intensity-modulated radiation therapy with concurrent chemotherapy for locally advanced cervical and upper thoracic esophageal cancer

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recurrences and 2 had distant metastases, 3 survived with no evidence of disease. After treatment, 2 patients developed esophageal stricture requiring frequent dilation and 1 patient developed tracheal-esophageal fistula.

CONCLUSION: Concurrent IMRT and chemotherapy resulted in an excellent early response in patients with locally advanced cervical and upper thoracic esophageal cancer. However, local and distant recurrence and toxicity remain to be a problem. Innovative approaches are needed to improve the outcome.

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Key words: Esophageal cancer; Intensity-modulated radiation therapy; Chemotherapy

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Abstract

AIM: To evaluate the dosimetry, efficacy and toxicity of intensity-modulated radiation therapy (IMRT) and concurrent chemotherapy for patients with locally advanced cervical and upper thoracic esophageal cancer.

METHODS: A retrospective study was performed on 7 patients who were definitively treated with IMRT and concurrent chemotherapy. Patients who did not receive IMRT radiation and concurrent chemotherapy were not included in this analysis. IMRT plans were evaluated to assess the tumor coverage and normal tissue avoidance. Treatment response was evaluated and toxicities were assessed.

RESULTS: Five- to nine-beam IMRT were used to deliver a total dose of 59.4-66 Gy (median: 64.8 Gy) to the primary tumor with 6-MV photons. The minimum dose received by the planning tumor volume (PTV) of the gross tumor volume boost was 91.2%-98.2% of the prescription dose (standard deviation [SD]: 3.7%-5.7%). The minimum dose received by the PTV of the clinical tumor volume was 93.8%-104.8% (SD: 4.3%-11.1%) of the prescribed dose. With a median follow-up of 15 mo (range: 3-21 mo), all 6 evaluable patients achieved complete response. Of them, 2 developed local

INTRODUCTION

Cervical esophageal cancer occurs rarely and accounts for only 2%-10% of all esophageal carcinomas in the United States^[1]. Surgery, an option only for patients with early-stage tumors, generally requires a total laryngopharyngoesophagectomy with reconstruction, an operation often leads to considerable dysfunction. The 5-year overall survival rate was only 16%-28% in two studies of patients treated with curative surgery^[2,3]. Unfortunately, surgical resection is not an appropriate treatment for those with locally advanced tumors, because it is difficult to achieve a clear margin. Radiotherapy alone, which does preserve laryngeal function, produces poor results, as evidenced by two studies of patients treated with definitive radiotherapy alone, showing the 5-year overall survival rate of only 14%-25%^[4,5].

Concurrent chemoradiotherapy is now standard treatment for locally advanced esophageal cancer, based on the results of an intergroup randomized controlled trial

(RTOG 8501)^[6]. Four studies examined the results from RTOG trial among small cohorts of cervical esophageal cancer patients who were treated with concurrent chemoradiotherapy^[7-10]. The 5-year survival rate was 55% in one^[9], and the 10-year survival rate was 27% in another^[8].

As to radiotherapy, treatment planning for cervical esophageal cancer is challenging partly because of the anatomical structures involved. The cervical esophagus lies in a close proximity to the spinal cord, courses through the lower neck and upper thorax with drastic change of contour and diameter of the anatomy; and the lymph nodes at risk must be incorporated into the irregular treatment volume.

Evidence suggested that, in these patients, high-dose radiotherapy results in better local control and survival compared with low-dose radiotherapy^[11]. However, high doses of radiotherapy may be associated with potentially high risk of complication because of the adjacent critical structure involved in the high radiation region, such as spinal cord.

Intensity-modulated radiation therapy (IMRT) is a novel approach to the planning and delivery of radiation therapy. Numerous investigators have demonstrated the benefits of IMRT planning in a variety of tumor sites in terms of the feasibility of normal tissue sparing^[12-14] and the delivery of higher radiation doses than conventional doses^[15,16]. Another advantage of IMRT is its ability to deliver differentiated dose to various structure during same fraction dose irradiation, thus allows to deliver a higher dose to gross tumor and lower dose to subclinical disease during same session of beam deliver, as commonly described as "simultaneous integrated boost (SIB)".

In two recent studies of esophageal cancer, IMRT plans were found to be superior to three-dimensional conformal radiation therapy (3D-CRT) plans in terms of dose conformity, homogeneity, and sparing of critical normal structure, such as the spinal cord and lung^[17,18]. An another study demonstrated that IMRT plans produced better dosimetric results than 3D-CRT in patients with cervical esophageal cancer^[19]. However, there is no clinical treatment result reported on patients with cervical esophageal cancer who were treated with IMRT.

At the University of Texas MD Anderson Cancer Center, we have used IMRT concurrently with chemotherapy for all patients with cervical esophageal cancer and for some with upper thoracic esophageal cancer since August 2002. We performed a retrospective study to evaluate the dosimetric considerations, early response and toxicity of this group of patients.

MATERIALS AND METHODS

Patient population

In this retrospective study, conducted from August 1, 2002 through December 31, 2004, 7 patients were selected according to the following inclusion criteria: newly diagnosed locally advanced cervical and upper thoracic esophageal cancer, definitive concurrent IMRT with chemotherapy treatment at the University of Texas MD Anderson Cancer Center, recoverable treatment plan

available. Patients were excluded if they had a distant metastasis or had esophagectomy or previously had radiotherapy in the neck or thoracic region. This study was approved by our Institutional Review Board, and informed consent for radiation therapy from all patients was taken. HIPPA compliance was enforced.

Pretreatment evaluation

For their pretreatment evaluation, all patients underwent computed tomography (CT) of the neck, chest, and abdomen, positron emission tomography (PET)-CT, and endoscopy and biopsy of the esophagus. Five patients underwent endoscopic sonography of the esophagus; the other 2 patients did not undergo endoscopic ultrasound examination due to severe obstruction of the esophagus by tumor; instead, they underwent bronchoscopy and PET-CT, which confirmed that they had T4N0 and T4N1 disease. In total, 6 of the 7 patients underwent bronchoscopy. The 1997 American Joint Committee on Cancer guidelines for the staging of cancer were used to classify tumor stage. Tumor stage was determined by endoscopic sonography and bronchoscopy findings, nodal stage was determined by endoscopic sonography or PET-CT, and metastasis stage was determined by PET-CT.

Chemotherapy

The induction chemotherapy consisted of two cycles of weekly carboplatin and paclitaxel ($n = 1$), given before chemoradiation. The concurrent chemotherapy consisted of continuous infusion of 5-fluorouracil (5-FU) (1000 mg/m²) on d 1 to 4 and 29 to 32 and cisplatin (75 mg/m²) on d 1 and 29 ($n = 2$), continuous infusion of 5-FU (700 mg/m²) on d 1 to 5 and 29 to 33 and cisplatin (75 mg/m²) on d 1 and 29 ($n = 1$), continuous infusion of 5-FU (300 mg/m²) on Monday to Friday for 5 wk and paclitaxel (45-50 mg/m²) weekly ($n = 2$), carboplatin area under the curve (AUC) 2 twice weekly and paclitaxel (30 mg/m²) weekly for 5 wk ($n = 1$), or carboplatin AUC 1 once weekly and docetaxel (20 mg/m²) weekly and continuous infusion of 5-FU (200 mg/m²) on Monday to Friday for 5 wk ($n = 1$).

Radiotherapy

All patients underwent CT simulation in a supine position with their arms by their sides; the CT images were taken at a 3-mm thickness throughout the entire neck and thorax. Four of the patients were immobilized with a head and neck/upper thoracic thermoplastic mask, and three with a vacuum-locked cradle. The gross tumor volume (GTV), clinical target volume (CTV), planning target volume (PTV), spinal cord, and lung parenchyma were outlined on each image. The GTV was defined as any visible tumor on the image. The CTV was defined as the GTV plus a 2- to 5-cm margin superior to the highest extension of the tumor and a 4- to 5-cm margin inferior to the lowest extension of the tumor with a 2-cm radial margin. Uninvolved bony structure and lung tissue were kept outside the CTV. The PTV was defined as the CTV plus a 5-mm margin. For patients in whom the SIB was used, the GTV boost was defined as the initial GTV plus a 1.5-cm surrounding margin. A 5-mm margin around normal structures, such as

the spinal cord and lung, was also added for the planning organ-at-risk volume (PRV).

The inverse IMRT plans for 4 of the 7 patients were created using Corvus software (v4.0) (Corvus, Nomos Inc., Sewickley, PA) and for the other 3 using Pinnacle (v6.2) software (Philips Radiation Oncology Systems, Andover, MA). All treatment plans used heterogeneity correction and were delivered with 6-MV photons. The mean dose, dose range, and standard deviation (SD) of the PTV (for GTV boost and CTV) were calculated. The minimum dose to the PTV was defined as the dose to the coldest 1% of target volume, and the maximum dose was defined as the dose to the hottest 1% of target volume. The SD percentage of the PTV was the SD dose to the PTV divided by the prescription dose to the PTV. The maximum dose to the spinal cord was defined as that received by 1 cm³ of the volume. The mean dose and the lung V₂₀ (volume of lung receiving ≥ 20 Gy) for the total lung were calculated. The lung V₂₀ < 40% and the maximum spinal cord dose of 45 Gy were considered to be acceptable for the IMRT plan. The pretreatment dosimetric quality assurance procedure and a test run were performed before the start of radiotherapy. Radiotherapy was delivered in a step-and-shoot mode with multi-leaf collimators. Portal films were obtained weekly to ensure the correct set-up.

Toxicity assessment

Acute toxicity levels were assessed weekly with complete blood cell counts and examinations for esophagitis and skin reactions during the concurrent chemoradiotherapy. Esophageal toxicity was defined as late if it occurred more than 90 d after radiotherapy. Radiation pneumonitis was defined as acute if it occurred within 6 mo after radiotherapy. All toxic effects were assessed using RTOG criteria^[20].

Follow-up evaluation

The following evaluations were performed 1 mo after treatment, every 3 mo for the first 2 years, and every 6 mo thereafter: physical examination, complete blood cell count, blood chemistry tests, an endoscopic examination, an esophageal biopsy, and scans of the neck, chest, and abdomen by CT, PET-CT, or both. Endoscopic sonography of the esophagus was performed in two of the patients to evaluate lymph node response.

Response assessment

A complete response (CR) of the primary tumor was defined as: (1) endoscopic examination was negative for all visible tumors and biopsy was negative for tumor cells lasted for ≥ 4 wk, and (2) no evidence of abnormal hypermetabolism on PET-CT scan. The response of the metastatic lymph nodes was assessed by CT, PET-CT, or endoscopic sonography of the esophagus. A CR was defined as the complete disappearance of all measurable and assessable disease for ≥ 4 wk.

Statistical analysis

The survival was calculated from the date of diagnosis.

RESULTS

Patients' characteristics

The median time of follow-up was 15 mo (range: 3-21 mo). Of the 7 patients included in this study, 6 were men. The Karnofsky performance status scores ranged from 70 to 90, and their ages ranged from 52 to 78 years. Six patients had cervical esophageal squamous cell carcinoma, and 1 patient had upper thoracic esophageal adenocarcinoma. At the time of presentation, 2 patients tolerated solid foods, 2 tolerated soft foods, and 3 tolerated only liquid diet. Three patients had lost more than 10% of their weight in the 3 mo before treatment.

Patients had locally advanced disease as follows: 3 patients had biopsy-proven tracheal invasion by tumor through bronchoscopy examination, rendered them as T₄ stage; one of them had 60% tracheal obstruction with a tracheal-esophageal (TE) fistula and had a tracheal stent placement before chemoradiation treatment; 3 patients had T₃ and 1 had T₂N₁ stage tumors. According to the endoscopic and PET-CT findings, the tumor length ranged from 3 to 8 cm. In 6 patients, a percutaneous gastrostomy or jejunostomy feeding tube was placed prior to the start of radiotherapy because of severe dysphagia, poor nutritional status, or a high risk of aspiration. The characteristics of the patients, tumor, and treatment are summarized in Table 1.

Dosimetric considerations

The primary tumor was irradiated with a total dose of 59.4-66 Gy given in 1.8-2.31 Gy/fraction. Three patients with cervical esophageal cancer also received prophylactic irradiation to the bilateral supraclavicular regions to a total dose of 46-60 Gy at 1.36-1.8 Gy/fraction. One patient with cervical esophageal cancer received bilateral supraclavicular and neck irradiation to a total dose of 56 Gy at 1.7 Gy/fraction because of small lymph node metastasis in the bilateral lower neck diagnosed by PET-CT. Three patients with cervical esophageal cancer also received irradiation to the superior mediastinum to a total dose of 56-66 Gy at 1.7-2.0 Gy/fraction. The SIB technique was used in 5 of the 6 patients with cervical esophageal cancer. In the other 2 patients, the GTV and CTV received the same fraction dose and total dose, since lymph node region was not prophylactically treated. Table 1 also shows the radiation treatment characteristics. Five, seven, eight, or nine coplanar beams with different gantry angles were used in the 7 patients. The total number of beam segments and monitor units (MU) was 218-559 and 21 252-40 812 for Corvus plans, and 198-417 and 27 423-42 504 for Pinnacle plans, respectively (Table 2). The daily treatment time was about 10-20 min.

Table 3 summarizes the dosimetric parameters of IMRT plans for the 7 patients. The median GTV was 78 cm³ (range: 17-229 cm³). The median GTV dose was 64.8 Gy (range: 59.4-66 Gy), and the median CTV dose was 56 Gy (range: 50.4-66 Gy). The minimum dose received by the PTV of boost GTV ranged from 91.2 to 98.2% (SD: 3.7%-5.7%) of the prescribed dose. The minimum dose received by the PTV of CTV ranged from 93.8% to 104.8% (SD: 4.3%-11.1%) of the prescribed dose. The V₂₀

Table 1 Patient and treatment characteristics for the 7 patients with cervical and upper thoracic esophageal cancer

Patient No.	Tumor distance from UES (cm)	Stage	Induction chemotherapy	Concurrent chemotherapy	XRT dose (GTV)	XRT dose (CTV)	XRT dose (supraclavical regions)
1	2	T ₃ N ₁ M ₀		5-FU, CDDP	64.8 Gy (2.31 Gy/f)	50.4 Gy (1.8 Gy/f)	50.4 Gy (1.8 Gy/f)
2	0	T ₂ N ₁ M ₀		5-FU, paclitaxel	59.9 Gy, (2.14 Gy/f)	50.4 Gy (1.8 Gy/f)	0
3	0	T ₃ N ₁ M ₀		5-FU, CDDP	64.8 Gy (1.8 Gy/f × 8, 2.21 Gy × 21)	51.7 Gy (1.53 Gy/f × 8, 1.88 Gy × 21)	46 Gy (1.36 Gy/f × 8, 1.67 Gy × 21)
4	6	T ₃ N ₀ M ₀		5-FU, paclitaxel	59.4 Gy (1.8 Gy/f)	59.4 Gy (1.8 Gy/f)	0
5	0	T ₄ N ₁ M ₀		5-FU, CBP, docetaxel	66 Gy (2.0 Gy/f)	66 Gy (2.0 Gy/f)	0
6	2.5	T ₄ N ₁ M ₀	CBP, paclitaxel	CBP, paclitaxel	66 Gy (2.0 Gy/f)	56 Gy (1.7 Gy/f)	56 Gy (1.7 Gy/f)
7	0	T ₄ N ₀ M ₀		5-FU, CDDP	66 Gy (2.0 Gy/f)	60 Gy (1.8 Gy/f)	60 Gy (1.8 Gy/f)

CBP: Carboplatin; CDDP: Cisplatin; CTV: Clinical tumor volume; GTV: Gross tumor volume; f: Fraction; UES: Upper esophageal sphincter; XRT: Radiotherapy

to the bilateral lung was 10.3%-36.0% in all patients, and the lung mean dose was 6.6-17.6 Gy (median: 12.6 Gy). The maximum dose to the spinal cord ranged from 37.2 to 45.8 Gy. The dose distribution and dose volume histogram (DVH) for patient number 7 are shown in Figures 1 and 2 using pinnacle planning system.

Tumor response and failure

One patient with cervical esophageal cancer, whose general condition gradually deteriorated, experienced excessive mucus production, weight loss and fever, and decided to begin home hospice care after 2 wk of radiotherapy, died of disease 4 mo after diagnosis. Thus, this patient's response was not evaluable and was not included in this analysis.

The other 6 patients all achieved a CR in the primary tumor and lymph node areas after concurrent chemoradiotherapy. The response was assessed by endoscopic biopsy for all patients, 4 of them also had PET-CT. Two patients had no evidence of disease (NED) at 13 and 17 mo. Two patients developed local recurrence in the esophagus 4 and 6 mo after treatment, one of them was successfully treated with salvage photodynamic therapy and had NED at 21 mo and the other died of disease at 15 mo. One patient had lung metastasis 7 mo after treatment and was alive with disease at 17 mo. One patient had both lymph nodes recurrence in the neck and soft tissue metastasis in the left thigh 11 mo after treatment and died of disease. Table 4 shows the tumor response and failure.

Toxicity

Acute major toxic effects included myelosuppression, dermatitis, and esophagitis. Myelosuppression occurred in 2 patients: 1 had grade 3 and another had grade 4 leukopenia. Three of the patients who had been immobilized with a thermoplastic mask experienced grade 3 skin reactions in the neck, and 1 of these required a 3-d treatment interruption. The patient who had a T4 tumor with a tracheoesophageal (TE) fistula and tracheal stent placement before chemoradiation developed grade 4 esophagitis 1 mo after treatment and had an esophageal stent placed 4 mo after treatment. Another patient experienced grade 4 late esophageal toxicity (TE

Table 2 Beam arrangement of IMRT plans for the 7 patients with cervical and upper thoracic esophageal cancer

Number of beams	Gantry angles	Number of segments	Number of monitor units per fraction	Total fraction	Total monitor units
7	0, 25, 65, 141, 212, 295, 335	315	1395	28	39 060
5	40, 70, 220, 240, 290	218	759	28	21 252
9	0, 18, 35, 70, 150, 225, 295, 320, 340	559	1248 and 1468	8 + 21	40 812
7	0, 30, 60, 105, 260, 300, 330	270	749	33	24 717
5	0, 50, 120, 240, 300	198	831	33	27 423
8	12, 36, 60, 135, 225, 300, 324, 348	332	1288	33	42 504
9	0, 40, 70, 130, 160, 200, 230, 290, 320	417	972	33	32 076

fistula) 7 mo after radiotherapy and had an esophageal stent placed. Two patients experienced grade 3 late esophageal toxicity (benign esophageal strictures requiring esophageal dilatation one to four times) 4 mo and 10 mo after radiotherapy. No patient had symptomatic radiation pneumonitis, although 2 patients had radiographic changes in their irradiated lung. No patients lost more than 10% of their weight during chemoradiotherapy. No patient experienced radiation myelitis. All except 1 patient completed radiotherapy without interruption in 37-44 d. Radiotherapy was interrupted for three day in 1 patient due to grade 3 skin toxicity and radiation was completed in 50 d. Table 4 summarizes the toxicity of chemoradiotherapy.

DISCUSSION

No consensus has been reached as to the optimal radiation technique and target volume delineation for treating cervical esophageal cancer, and a survey conducted in Canada has come up with different opinions from radiation oncologists^[21,22]. IMRT has potential benefit for treating cervical esophageal cancer because of the complexity of anatomy in this region. Separate IMRT plans designed for the initial large-field treatment and the subsequent boost

Table 3 Dosimetric results of IMRT for 7 patients with cervical and upper thoracic esophageal cancer

Patient No.	Dose (Gy)	GTV volume (cm ³)	PTV mean dose (Gy)	PTV min %	PTV max %	PTV SD %	Lung V ₂₀ (Gy)	Lung mean dose (Gy)	Spinal cord max (Gy)
1	64.8 (GTV)	58	70.5	96.5	117.9	4.9	18.9	12.0	42.8
	50.4 (CTV)		66.4	104.8	149.6	11.1			
	50.4 (sup)		61.3	101.6	149.2	11.9			
2	59.9 (GTV)	53	65.0	91.2	122.9	6.0	28.6	13.4	25.7
	50.4 (CTV)		58.7	103.9	139.4	6.6			
3	64.8 (GTV)	85	68.6	97.8	117.5	3.7	10.3	6.6	37.2
	51.7 (CTV)		62.3	93.8	121.2	9.2			
	46 (sup)		54.2	100	143	8.1			
4	59.4 (GTV)	17	65.0	97.1	119.1	4.3	24.2	11.6	39.2
5	66 (GTV)	78	67.3	97.9	109.2	4.9	26.8	12.6	45.8
6	66 (GTV)	229	68.0	98.2	110.9	4.8	21.9	7.7	40.2
	56 (CTV + neck)		59.8	101.1	122.9	9.4			
	66 (GTC)		66.4	99.4	111.8	5.7			
	60 (CTV)		65.7	97	121.6	6.7			
7	60 (sup)	169	67.1	105	116.8	5.4	36	17.6	41.8

GTV: Gross tumor volume; CTV: Clinical target volume; PTV: Planning target volume; sup: Bilateral supraclavicular region; min: Minimum; max: Maximum; SD: Standard deviation.

Table 4 Treatment response and toxicities for the 7 patients with cervical and upper thoracic esophageal cancer

Patient No.	Response (primary and node)	Relapse	Life status	Leucopenia (grade)	Dermatitis (grade)	Esophagitis (grade)	Pneumonitis (grade)	Late esophageal toxicity (grade)
1	CR	LR	DOD	1	1	2	0	0
2	Not evaluable	Not evaluable	DOD	1	3	2	0	not evaluable
3	CR	NED	Alive NED	0	1	2	0	0
4	CR	LR	Alive NED	3	1	1	0	3
5	CR	NED	Alive NED	0	3	1	0	3
6	CR	DM and node	DOD	0	3	2	0	0
7	CR	DM	Alive with disease	4	2	4	0	4

LR: Local recurrence in the esophagus; NED: No evidence of disease; DM: Distant metastasis; DOD: Died of disease

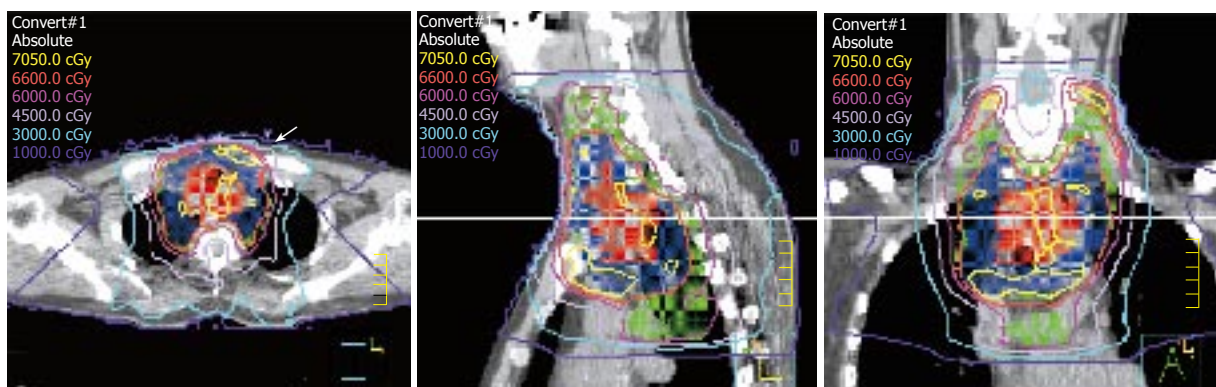


Figure 1 Dose distribution of the IMRT plan for patient number 7. The GTV (red), boost PTV (blue) and PTV (green) are shown on the contours.

treatment are referred as two-phase IMRT strategies. On the other hand, the term “simultaneous integrated boost” (SIB) is used to define treatment plan that delivers differentiated doses to different targets during a single fraction dose delivery as follows: one IMRT plan simultaneously delivers different dose levels to different

targets in a single treatment session, which results in the primary target (e.g., palpable or visible disease or GTV) and the secondary target (e.g., regions at risk for microscopic disease or CTV) being treated to different dose levels in each and same treatment fraction. Using this approach, the fractional dose delivered to gross tumor

can be increased, while, at the same time, the radiation doses and dose schedules known to be adequate for tumor control in marginal tissues and clinically uninvolved lymph nodes are preserved. Thus, the SIB-IMRT has been considered a novel method for accelerated fractionation therapy in controlling gross disease through dose-per-fraction escalation. It can shorten overall treatment time, which is preferable for treating tumors with rapid repopulation. It has the ability to deliver large IMRT fields, often required for simultaneous treatments of all target volumes, by splitting them and dynamically feathering their junctions for greater accuracy^[23]. Therefore, it is an easy, efficient, and less error-prone method of planning and delivery, because it allows the same plan to be used for the entire course of treatment.

Since the large-field and boost doses are delivered in the same number of fractions, one must consider the radiobiological consequences of different fraction sizes for the gross disease, regions of microscopic spread, and electively treated lymph nodes. One can select the conventional 2 Gy per fraction for the gross disease for an SIB strategy, but that might lead to a significantly lower dose per fraction to volumes of microscopic disease and electively treated lymph nodes. On the other hand, one can choose to deliver 2 Gy per fraction to the lower and intermediate dose volumes, but this would require a high dose per fraction, as much as 2.5 Gy or more per fraction, to the gross disease. The latter scheme may have the advantage of shortening the treatment duration and a potential for improvement in local control but at an increased risk of injury to the embedded normal tissues.

In our study, the IMRT plans showed good homogeneity and conformity and the treatment time was acceptable. SIB-IMRT was used in 5 of 7 patients, which made the treatment course simple. Several researchers reported that SIB-IMRT is superior to two-phase IMRT (sequential IMRT) in terms of conformity of dose distribution within the target volume and the sparing normal tissue for head and neck cancer^[24, 25], lung^[25, 26] and prostate cancers^[25]. The SIB-IMRT also has some advantage over 3-dimensional conformal radiotherapy regarding homogeneity of tumor dose and reduction of the dose to normal tissue for cervical esophageal cancer^[19] and malignant glioma^[27, 28].

Defining target volume delineation for IMRT plan, especially a consistent CTV delineation, is challenging because current imaging techniques are not capable of directly detecting subclinical tumor involvement and also because the patterns of failure is not clear. It is difficult to differentiate if the local tumor recurrence is due to a geographic miss or persistent disease. The tendency for esophageal cancer to be multicentric or to present with submucosal skip metastasis supports the use of generous proximal and distal margins for treatment. In the RTOG 8501 trial^[6], the entire esophagus was included in the radiation portals, but the toxic effects were severe. In the subsequent RTOG 9405 study^[29], 5-cm proximal and distal margins and a 2-cm radial margin were added around the GTV which is effective and has been accepted as a standard in most institutions in the United States.

We used slightly smaller proximal than distal margins in our study, because of our concern about increased

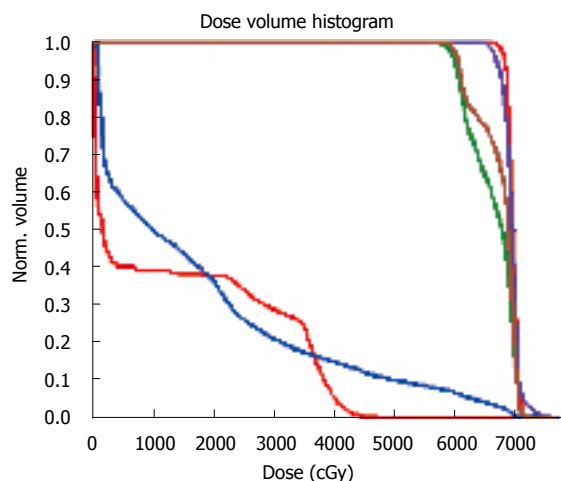


Figure 2 Dose volume histogram (DVH) of the IMRT for patient number 7, showing DVH curves for GTV, boost PTV, CTV, PTV, total lung and spinal cord (from right to left). Norm. Volume = Normalized volume.

toxic effects on the pharynx. Prophylactic irradiation of the supraclavicular and superior mediastinal nodes could decrease the risk of nodal relapse without greatly increasing the toxic effects. In the RTOG 94-05 study^[29], boost GTV was defined as a 2-cm margin around the initial GTV; we used the same boost GTV expansion in our study (1.5-cm margin from GTV to boost GTV and another 5 mm added for PTV).

This is the first study that we are aware of that reported the clinical result for esophageal cancer patients treated with IMRT. In this study, all 6 evaluable patients achieved complete response both in the primary tumor and lymph node sites. Although the early response result was very encouraging for this small group of locally advanced tumors, 2 patients developed local recurrences and another 2 had distant metastases. Only 3 patients survived with no evidence of disease during a median time of 13 mo follow-up. Although some clinical data on cervical and upper thoracic esophageal cancer patients who were treated with concurrent chemotherapy and radiotherapy with conventional or 3-dimensional conformal techniques have been published, the data are sparse because the disease is rare and the published series have consisted of relatively few patients. For example, Ampil *et al*^[7] reported that only 2 of their 6 patients achieved CR when conventional radiation techniques were used to deliver a total dose ranging from 36 to 64 Gy at 2.0 Gy/fraction. Burmeister *et al*^[9] reported that 31 of their 34 patients had CR after a total dose delivered by 3-dimensional conformal radiation that ranged from 50.4 Gy in 20 fractions to 65 Gy in 33 fractions. The 5-year survival rate in this group was 55%. However, only 3 patients in this group had locally advanced disease; most had early disease. Moreover, Bidoli *et al*^[8] reported that 37 of their 58 patients (29 of whom had cervical esophageal cancer) experienced a CR after having been given 50 Gy in 25 fractions with conventional radiation techniques. The 10-year survival rate was 27% for the 29 patients with cervical esophageal cancer. We await the maturity of our study to report the long-term outcome.

Three patients experienced severe skin toxicity, probably caused by the use of the mask and the lack of effort to avoid irradiating the skin during IMRT planning. In a study by Burmeister *et al*^[9] with 3-dimensional conformal radiotherapy, a moderate-to-severe skin reaction occurred in most patients on the neck. IMRT technique has the potential to reduce skin toxicity if skin was defined as an avoidance structure. From our experience, to reduce toxic effects to the skin, we need to use a cradle instead of a mask to immobilize patient, also consider skin as an avoidance structure during IMRT planning.

The development of acute esophagitis during and after radiotherapy is usually unavoidable. Fortunately, no patient lost more than 10% of body weight or had to have treatment interrupted because of esophagitis, probably because of the intensive supportive and nutritional therapy received through a gastrostomy or jejunostomy tube. Therefore, the prophylactic use of such tubes is recommended for patients who have severe dysphagia or poor nutritional status prior to the initiation of chemoradiation.

Development of late esophageal toxic effects is a concern for esophageal cancer survivors. An esophageal stricture caused by fibrosis of the esophagus, usually at the site of tumor, was mainly due to the high total radiation dose delivered^[30]. In this study, 3 of 4 surviving patients had esophageal stricture or fistula. Burmeister *et al*^[9] demonstrated that in 34 patients treated with 3-dimensional conformal radiotherapy, strictures were the most notable late effect of the therapy: 11 patients developed mild strictures that did not require dilation, 4 patients developed strictures requiring repeated dilation, and 1 patient developed a severe stricture that did not respond to dilation. In the patient with a severe stricture, the attempted corrective surgery failed and resulted in death of the patient. In addition, the local tumor recurrence remains to be a concern even though relatively high radiation dose was used for this group of patients. The degree of dose escalation by IMRT was limited by long-term toxicity, such as esophageal strictures and fistula. For patients who had their local diseases well-controlled, development of distant metastases became a major pattern of failure.

Therefore, new approaches besides IMRT are needed. One possible way is to use induction chemotherapy. Theoretically, induction chemotherapy could treat the occult micrometastatic disease upfront and may lead to a decrease in the incidence of distant metastases; in addition, induction chemotherapy could be effective in shrinking the primary tumor so that only moderate radiation dose is required to control the local disease, while reducing the late esophageal toxicity. Stuschke *et al*^[10] reported their results of induction chemotherapy plus concurrent chemoradiotherapy (a total dose of 60 to 65 Gy in 6 wk delivered by 3D-CRT) for 22 patients with upper and midthoracic locally advanced squamous cell carcinomas. Locoregional recurrences as one site of first relapse were observed in 12 patients and distant metastases as one site of first relapse occurred in 4 patients. Seven long-term survivors had a good swallowing function. Induction chemotherapy results in a major response rate of 45%

and response to induction chemotherapy is strongly associated with long-term survival and locoregional tumor control. No long-term survivors are found among non-responders to induction chemotherapy. The criterion good response to induction chemotherapy can be used to select patients for high-dose radiotherapy. Another way is molecular targeting agents. Epidermal growth factor inhibitor (EGFR), such as cetuximab, in combination with radiotherapy yielded positive results in treating head and neck squamous cell carcinoma^[31]. It is perhaps worthwhile to test EGFR in treatment of cervical and upper thoracic esophageal cancer, because squamous cell carcinoma is the major histology.

In conclusion, concurrent IMRT and chemotherapy resulted in an encouraging tumor response in patients with locally advanced cervical and upper thoracic esophageal cancer. However, local and distant recurrence, and late toxicity remain to be a challenge in the management of this disease. Additional innovative treatment modalities are needed to improve the outcome. All patients with cervical and upper esophageal tumor should be registered and treated in a prospective study with consistent chemotherapy and IMRT dose schedule, and possibly target therapy.

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Prevalence of *H pylori* associated 'high risk gastritis' for development of gastric cancer in patients with normal endoscopic findings

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gastric cancer development-only a proper follow-up can provide this information.

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Key words: *H pylori*; Gastric cancer; Gastritis

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Abstract

AIM: To investigate the prevalence of *H pylori* associated corpus-predominant gastritis (CPG) or pangastritis, severe atrophy, and intestinal metaplasia (IM) in patients without any significant abnormal findings during upper-GI endoscopy.

METHODS: Gastric biopsies from 3548 patients were obtained during upper GI-endoscopy in a 4-year period. Two biopsies from antrum and corpus were histologically assessed according to the updated Sydney-System. Eight hundred and forty-five patients (mean age 54.8 ± 2.8 years) with *H pylori* infection and no peptic ulcer or abnormal gross findings in the stomach were identified and analyzed according to gastritis phenotypes using different scoring systems.

RESULTS: The prevalence of severe *H pylori* associated changes like pangastritis, CPG, IM, and severe atrophy increased with age, reaching a level of 20% in patients of the age group over 45 years. No differences in frequencies between genders were observed. The prevalence of IM had the highest increase, being 4-fold higher at the age of 65 years versus in individuals less than 45 years.

CONCLUSION: The prevalence of gastritis featuring at risk for cancer development increases with age. These findings reinforce the necessity for the histological assessment, even in subjects with normal endoscopic appearance. The age-dependent increase in prevalence of severe histopathological changes in gastric mucosa, however, does not allow estimating the individual risk for

INTRODUCTION

H pylori infection is an established risk factor for development of gastric cancer^[1,2]. According to the model of carcinogenesis of the intestinal type adenocarcinoma proposed by Correa, the multi-step development starts from the condition of a chronic active gastritis, followed by glandular atrophy, intestinal metaplasia, dysplasia, and finally gastric adenocarcinoma^[3]. The risk for gastric cancer also increases in the presence of corpus-predominant gastritis as well as a pangastritis^[4]. Since only a small subset of *H pylori* infected subjects will develop gastric cancer, from a clinical point of view it is a major difficulty to identify those patients with *H pylori* infection who are at a higher risk to develop gastric cancer. Therefore there is a need to identify patients, who have these advanced changes in gastric mucosa. The combination of these risk factors (gastric cancer risk index) has been proven to be a simple way to better estimate the risk for gastric cancer development^[5,6]. The gastric cancer risk index has been proven to be independently increased in both histological types of gastric cancer: the intestinal as well as the diffuse type.

The aim of the present study was to estimate the prevalence of advanced histopathological changes in gastric mucosa in patients with normal appearance of gastric mucosa during upper GI-endoscopy.

MATERIALS AND METHODS

A total of 3548 first time upper GI-endoscopies were

Table 1 Age and sex distribution of *H. pylori* positive patients without gross pathology of gastric mucosa during upper GI-endoscopy

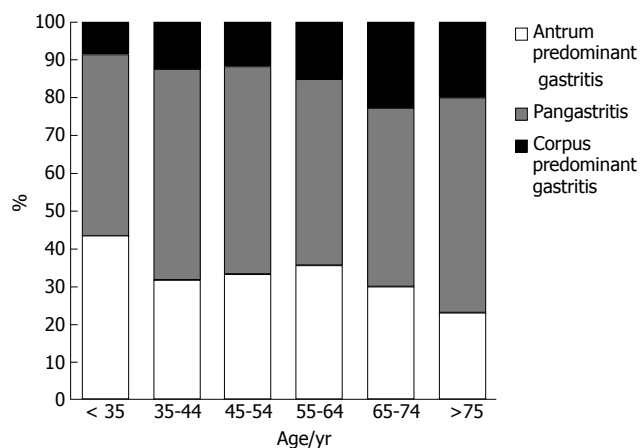
Endoscopic normal gastric mucosa with <i>H. pylori</i> infection	
Number (n)	845
Mean age (yr)	54.8 (18-87)
Sex	
Male	450/845 (53.2%)
Female	395/845 (46.8%)
Age (yr)	
< 35	114/845 (13.5%)
35-44	120/845 (14.2%)
45-54	151/845 (17.9%)
55-64	199/845 (23.6%)
65-74	166/845 (19.6%)
> 75	95/845 (11.2%)

performed in the Endoscopic Unit of the University of Magdeburg over a 4-year period. During this period, it was the policy in our department to obtain gastric specimens from all patients regardless of the appearance of the mucosa to establish the pattern of gastritis in those patients. Patients with contraindication to obtain gastric specimens or previous endoscopy with histological investigation were not included.

Around one half of the investigated patients were outpatients, and the other half hospitalized patients. After the investigation, an endoscopic report was entered in a computer-based database. Upper GI-endoscopy was performed by several experienced gastroenterologists. Two gastric specimens from the corpus and antrum, respectively, were taken and analysed histologically according to the Sydney-System in all patients. Histological results were documented in the same computer-based database. Histological examination was performed by different pathologists from the Institute of Pathology at the University of Magdeburg. All slides have been re-evaluated for the needs of this study by a single experienced gastrointestinal pathologist in a blinded way without being aware of clinical or endoscopic diagnoses and prior histological reports. The study was performed according to the guidelines of the Ethics Committee of the Medical Faculty of the University of Magdeburg and all patient data were made anonymous to protect patients' identity.

The database was retrospectively analyzed for those patients who had a normal endoscopic appearance of gastric mucosa or simply signs of gastritis or duodenitis. Thus all patients with gastric or duodenal ulcers, GI-bleeding, and upper GI-malignancy were excluded. The presence of reflux oesophagitis or hiatal hernia was not an exclusion criterion. Furthermore, patients known to be treated with proton-pump inhibitors or with indirect evidence for this treatment by histological observation of parietal cell hypertrophy were excluded. *H. pylori* infection was determined by positive culture, rapid urease test and/or histology.

We identified 845 *H. pylori* positive patients who fulfilled the predefined criteria. Age distribution and gender are

**Figure 1** Prevalence of different phenotypes of gastritis in patients with *H. pylori* infection in different age groups. With increasing age the number of patients with corpus-predominant gastritis increased significantly, whereas the antrum predominant gastritis decreased (Kruskal Wallis test; $P < 0.01$).

given in Table 1. In these patients the following parameters were calculated according to the histological examination: (1) Corpus-predominant gastritis^[4]: Higher degree of neutrophilic infiltration in the corpus compared to the antrum; (2) Pangastritis^[4]: Equal degree of neutrophilic infiltration in the corpus and in the antrum; (3) Antrum-predominant gastritis^[4]: Higher degree of neutrophilic infiltration in the antrum compared to the corpus; (4) Intestinal metaplasia^[4,7]: Absence or presence in any investigated specimen from antrum or corpus; (5) Severe atrophy^[4,8]: Severe loss of glands; not diagnosed in the antrum when only a few intestinal crypts were observed in the whole specimen; (6) Antrum and corpus gastritis score: Score by total sum of grade of gastritis (mild = 1, moderate = 2, marked = 3 infiltration with lymphocytes and plasma cells) and activity of gastritis (mild = 1, moderate = 2, marked = 3 infiltration with neutrophilic granulocytes) either in the antrum or in the corpus, a maximum of a sum of 6 points in the antrum and in the corpus for each individual person; (7) Gastric cancer risk index^[5,6]: 1 point scored for at least moderate infiltration with lymphocytes and plasma cells in the corpus and less or equal infiltration in the antrum, 1 point scored for at least moderate infiltration with neutrophilic granulocytes in the corpus and less or equal infiltration in the antrum, 1 point scored for the detection of intestinal metaplasia in the antrum or in the corpus, a maximum of 3 points for each individual person.

RESULTS

The frequency of corpus-predominant gastritis was significantly increased with age (Kruskal-Wallis test, $P < 0.01$). While in the group of patients of less than 35 years old only 8.8% had a corpus-predominant gastritis, and the number increased to 22.9% between the ages of 65-75 years (Figure 1). The highest increase was observed between the age groups of 45-54 years (13.8%) and 55-64 years (23.1%). The number of patients with pangastritis remained constant, whereas the frequency of antrum-predominant gastritis declined over time.

Table 2 Frequency of intestinal metaplasia (IM) in the gastric antrum and the corpus in patients without macroscopic pathology during upper GI-endoscopy

Age (yr)	Overall IM	IM only in the antrum	IM only in the corpus	IM in antrum and corpus
< 35	9/114 (7.9%)	1/114 (0.9%)	7/114 (6.1%)	1/114 (0.9%)
35-44	14/120 (11.7%)	2/120 (1.7%)	12/120 (10.0%)	0/120 (0.0%)
45-54	28/151 (18.5%)	7/151 (4.6%)	18/151 (11.9%)	3/151 (2.0%)
55-64	50/199 (25.1%)	7/199 (3.5%)	35/199 (17.6%)	8/199 (4.0%)
64-75	65/166 (39.2%)	6/166 (3.6%)	51/166 (30.7%)	8/166 (4.8%)
> 75	38/95 (40.0%)	6/95 (6.3%)	24/95 (25.2%)	8/95 (8.4%)
Differences between age groups	$P < 0.001$ (χ^2 -test)	$P < 0.001$ (χ^2 -test)	$P < 0.001$ (χ^2 -test)	$P < 0.001$ (χ^2 -test)

Table 3 Frequency of severe atrophy, antrum- and corpus-gastritis score in *H. pylori* positive patients in different age-groups in patients without macroscopic pathology during upper GI-endoscopy

Age (yr)	Severe atrophy	Antrum gastritis score	Corpus gastritis score
< 35	0/114 (0.0%)	3.36 \pm 0.90	2.49 \pm 1.17
35-44	2/120 (1.7%)	3.17 \pm 0.86	2.73 \pm 1.02
45-54	3/151 (2.0%)	3.32 \pm 1.04	2.85 \pm 1.04
55-64	4/199 (2.0%)	3.38 \pm 0.94	2.93 \pm 1.17
64-75	9/166 (5.4%)	3.20 \pm 0.92	2.98 \pm 1.08
> 75	9/95 (11.2%)	3.08 \pm 0.96	3.06 \pm 1.15
Differences between age groups	$P < 0.001$ (χ^2 -test)	NS (ANOVA)	$P < 0.001$ (ANOVA)

The frequencies of intestinal metaplasia in different age groups increased significantly in an almost linear pattern ($r^2 = 0.9585$, $P < 0.001$). The increase rate was 0.71% per year (95% CI 0.51-0.92). Nearly 40% of patients over 65 years had intestinal metaplasia (Table 2). For example, the relative risk for a patient at the age of 64-75 years was 5.0 (95% CI 2.6-9.6) times higher compared to a patient younger than 35 years. Intestinal metaplasia was more frequent in the corpus compared to the antrum (20.7% *vs* 6.7%), and those located only in the antrum were a rare event (3.4%).

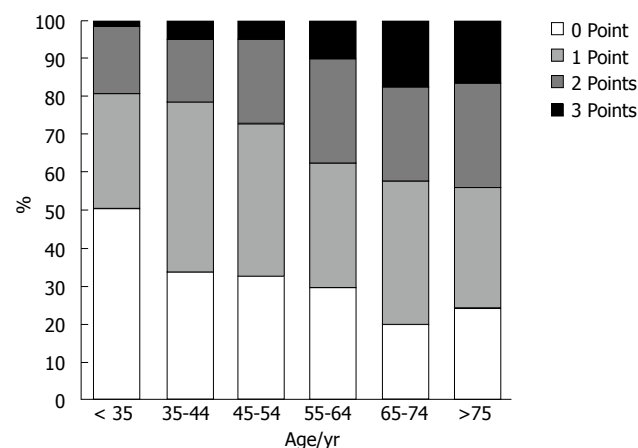
The frequencies of severe atrophy and the antrum/corpus gastritis score in the different age groups are provided in Table 3. Severe atrophy was close to 2% in the group younger than 65 years and increased up to 11.2% at the age over 75 years. The antrum gastritis score was almost equal in all age groups (mean 3.27 ± 0.95). The corpus gastritis score increased significantly with age [increase of 0.01 per year (95% CI 0.006-0.016)].

The gastric cancer risk index showed a significant shift to a higher index with age (Kruskal-Wallis test; $P < 0.0001$; Figure 2). At least 10% of the individuals older than 55 years had a score of 3 points, and more than 25% of them had a score of 2 points.

Comparison of the different scoring systems like gastric cancer index and phenotype of gastritis showed a significant correlation ($P < 0.001$, χ^2 -test; Table 4). This was expected because the phenotype of gastritis was included in the gastric cancer index. Nevertheless, the definitions for predominant gastritis were very different (see

Table 4 Comparison of gastric cancer risk index and phenotype of gastritis in 845 patients with *H. pylori* positive gastritis

Phenotype of gastritis	Gastric cancer risk index			
	0 point	1 point	2 points	3 points
Antrum-predominant gastritis	156	104	21	0
Pangastritis	107	178	113	36
Corpus-predominant gastritis	2	22	62	44

**Figure 2** Gastric cancer risk index in patients with *H. pylori* infection in different age groups. With increasing age the number of patients with 2 or 3 points increased significantly (Kruskal Wallis test; $P < 0.0001$).

METHODS). The group with the highest risk for gastric cancer according to Uemura *et al.*^[4] might be these with the corpus-predominant gastritis ($n = 130$), whereas the risk might be highest for those with a gastric cancer index of 3 ($n = 80$), and the overlapping group contained only 44 patients (26.5%).

DISCUSSION

Histological alterations of the gastric mucosa such as corpus-predominant gastritis, pangastritis, intestinal metaplasia or severe atrophy are proposed to carry an increased risk for gastric cancer in *H. pylori* infected individuals. In our study we observed a strong association of intestinal metaplasia, atrophy as well as corpus-predominant gastritis with increasing age. Our results represent the frequency of histopathological changes in a country with a lower gastric cancer prevalence compared to other parts of the world (i.e. China or Japan). This might be of importance for future investigational trials on this topic.

We investigated only patients with normal appearing gastric mucosa or no grossly visible changes at endoscopy. In such a setting, often a rapid urease test would be performed by most investigators to test for *H. pylori* only; therefore histopathological changes will not be diagnosed. However, our data clearly indicate that there is a need to obtain gastric specimens in routine endoscopy, even if the macroscopic appearance is to a greater or lesser extent normal. There is much evidence that several phenotypic changes in the gastric mucosa are associated with an

increased risk of gastric cancer. In a large prospective study from Japan^[4] only patients with *H pylori* infection developed gastric cancer. A closer look at the data revealed that certain histopathological findings increased the risk dramatically. In fact, the presence of intestinal metaplasia (RR 6.4), severe atrophy (RR 4.9), pangastritis (RR 15.6), and, most strikingly, corpus-predominant gastritis (RR 34.5) was associated with an increased risk for gastric cancer. An additional aspect in this study was the fact that about 60% of the patients who developed gastric cancer, had no initial pathological findings during upper GI-endoscopy and were classified as non-ulcer dyspepsia. It demonstrates the importance to obtain gastric specimen during upper GI-endoscopy even though the macroscopic findings are normal.

The optimal strategy for patients with risk lesions for gastric cancer has not been established. One option might be to eradicate *H pylori* infection. It is well known that corpus-predominant or pangastritis will be healed after successful eradication therapy; on the other hand, atrophy and intestinal metaplasia will persist constantly according to systematic review of the published literature^[9]. In addition, a large randomized trial from China indicates that the point of no return might be already achieved when atrophy or intestinal metaplasia are observable^[10]. In that trial, performed in more than 1600 healthy volunteers from Fujian Province, a significant reduction of gastric cancer incidence in an 8-year follow-up period by *H pylori* eradication was only observed in those patients without intestinal metaplasia or atrophy in the gastric mucosa in the beginning of the study. One possible conclusion from this study is that patients with those histopathological changes have to be included in an endoscopic surveillance program, but it is unknown which intervals are necessary and how cost-effective such a strategy might be. This will have to be the topic of future investigational trials.

The combination of histopathological features is a promising tool, since there is a clear need to identify those patients at risk for the development of gastric cancer. While the scoring system used in the trial by Ley *et al*^[11] is probably too complex to use in general, the gastric cancer index might be more promising to use in clinical practice. This score includes the type of gastritis as well as the presence of intestinal metaplasia and has been proven to be highly predictive for the presence of gastric carcinoma^[5]. We identified 80 (9.5%) out of 845 patients with normal endoscopic appearance and the highest possible gastric cancer risk score of 3. Especially in those patients an endoscopic surveillance might be necessary; however, the final proof of any improvement for the individual patient is yet missing.

In the trial of Uemura *et al*^[4] the highest risk was found in those patients with a corpus-predominant gastritis; nevertheless, a marked risk was observed in those with pangastritis, also. Pangastritis was very frequently

diagnosed in our population (51.3%). The significance of this finding therefore will have to be verified.

Our results indicate that there is a need to obtain gastric biopsies during upper GI-endoscopy, even if the macroscopic appearance is normal or without any gross pathology. The prevalence of gastritis associated with an increased risk for cancer development increases with age. The individual risks for gastric cancer development remain to be established by the follow-up. *H pylori* eradication therapy alone might not be sufficient enough to prevent gastric cancer in patients with advanced changes in the gastric mucosa. From the actual perspective, and with regard of the literature, interventions have to be implemented earlier, before advanced changes in gastric mucosa caused by *H pylori* occur or, more worthwhile, preventing being infected with *H pylori*.

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Genetic association of autoimmune hepatitis and human leucocyte antigen in German patients

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HLA. A clinical correlation, e.g. difference in severity or treatability of AIH type 1, has yet to be determined.

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Key words: Autoimmune hepatitis; Human leucocyte antigen; Immunogenetics

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Abstract

AIM: To report on our large German collective and updated data of 142 patients with autoimmune hepatitis (AIH) type 1.

METHODS: Key investigations performed were liver biopsy, serum autoantibodies as well as serum markers such as IgG and elevated transaminases. Antinuclear antigen (ANA) and smooth muscle antigen (SMA) autoantibodies characterized type 1 AIH. Type 3 (AIH) was solely characterized by the occurrence of soluble liver antigen/liver-pancreas antigen (SLA/LP) autoantibodies either with or without ANA or SMA autoantibodies.

RESULTS: Most prevalent HLAs were A2 (68 patients, 48%), B8 (63 patients, 44%), C7 (90 patients, 63%), DR3 (49 patients, 38%), DR4 (49 patients, 38%) and DQ2 (42 patients, 30%). Compared to the Italian and North American patients, we found fewer patients with a DQ2 subtype. Furthermore, the B8-DR3-DQ2 human leucocyte antigen (HLA) was also less prominent compared to the North American patients. However, prevalences of B8, DR3, DR4, DR7, DR11 and DR13 were comparable to the Italian and North American patients. Furthermore, we report on an additional subgroup of patients with SLA/LP positive AIH. Generally, in this subgroup of patients the same HLA subtypes were favoured as the AIH type 1.

CONCLUSION: Although HLA subtypes were comparable between these three collectives, the German patients were distinct from the Italian and North American patients with respect to DQ2 and from the North American patients with respect to B8-DR3-DQ2

INTRODUCTION

Autoimmune hepatitis (AIH) is characterized by portal lymphatic infiltrates on liver histology and in most patients with the occurrence of autoantibodies such as antinuclear, smooth muscle antibody-positive (ANA/SMA, type 1), liver-kidney microsomal antibody-positive (LKM-1), and soluble liver antigen/liver-pancreas antigen (SLA/LP) antibodies. Untreated, the disease usually runs an unfavorable course with 5 year survival rates of 50% and 10 year survival rates between 10% and 27%^[1,2]. However, in most patients the disease can be controlled with immunosuppressants such as prednisolone and azathioprine, resulting in an almost normal average life expectancy^[3].

Although the pathomechanism of the disease is still unknown, an underlying genetic predisposition has been suggested due to the fact that patients are predominantly of female gender (women to men ratio equals to approximately 6:1) and the association of the disease with certain human leucocyte antigens (HLAs). Muratori *et al*^[4] recently published an extensive study on two large populations, Italian and North American, and demonstrated a distinct genetic association of HLA with the disease. B8-DR3-DQ2 was reported to be the most frequent genotype in Italian patients with AIH type 1 but significantly less frequent in North American patients. In addition, a clear difference in occurrence of the DR4 genotype was demonstrated with fewer patients in Italy presenting with such a genotype. Furthermore, the C7, DR3, DR11, DR13, and DQ2 loci were investigated but no significant differences between

Table 1 Most prevalent HLA subtypes in German patients with autoimmune hepatitis type 1

HLA	AIH type I German patients
C7	90 (63%)
A2	68 (48%)
B8	63 (44%)
DR3	49 (38%)
DR4	49 (38%)
DQ2	42 (41%)

these two populations could be found.

In order to further elucidate these genetic associations and differences, we now report on the HLA antigens in our large German collective of 142 patients with AIH type 1 and compared it to published data, especially in the Italian and North American populations. In addition, we report on our small subgroup of 29 patients with SLA/LP positive AIH.

MATERIALS AND METHODS

Subjects

One hundred and forty-two consecutive patients with definite, autoantibody positive AIH type 1 and 29 consecutive patients with SLA/LP positive AIH, who had been referred to the Department of Internal Medicine I, Mainz University Hospital, were investigated. All patients lacked serological evidence of chronic viral hepatitis B and C by third-generation enzyme-linked immunoassay. There was no evidence for illicit drug abuse, excessive alcohol intake (> 4 oz/wk) or exposure to hepatotoxic drugs. Diagnosis of AIH was established according to the revised Scoring system described previously^[5].

Methods

Key investigations performed were liver biopsy, serum autoantibodies as well as serum markers such as IgG and elevated transaminases. Similar to Muratori's study^[4] and according to international standards, ANA and SMA autoantibodies characterized type 1 AIH. Type 3 (AIH) was solely characterized by the occurrence of SLA/LP autoantibodies either with or without ANA or SMA autoantibodies.

Of our 142 patients with AIH type 1, 119 were women (84%) and 23 were men (16%). Of these patients, 108 (76%) were positive for ANA, 101 (71%) for SMA autoantibodies, and 67 (47%) patients had both ANA and SMA autoantibodies. DR and DQ alleles were not examined in all patients. DR alleles were investigated in 129 patients and DQ in 103 patients. Of the 29 patients with AIH type 3, 22 were women (76%) and 7 were men (24%). Since HLA loci are thought to be genetically fixed and to be independent of age, serum markers or immune globulins, patients were not further characterized in that respect.

RESULTS

The most commonly found HLA subtypes in our patients

with AIH type 1 were C7 (90 patients, 63%), A2 (68 patients, 48%), B8 (63 patients, 44%), DR3 (49 patients, 38%), DR4 (49 patients, 38%) and DQ2 (42 patients, 30%) (Table 1). As significant differences had been demonstrated in the Italian and North American populations regarding the distribution of the B8-DR3-DQ2 and DR4 HLA subtypes, these subtypes were also analysed along with the additionally reported C7, DR7, DR11, DR13, and DQ2 loci. The B8-DR3-DQ2 subtype was identified in 28 (27%) German patients with AIH type 1. And 49 patients (38%) were tested positive for the DR4 locus. In addition, almost half of all patients with AIH type 1 were positive for the HLA subtype B8 (45%) and 38% for DR3 (Table 2). The C7 HLA subtype which was highly prevalent in Italian patients with AIH type 1 was also common in the German population with 90 patients (63%). Further HLA subtypes reported and compared in Italian and North American patients were less prevalent in the German population. DR11 was positive in 17 (13%) of patients. Also DR13 could be identified in 17 (13%) of patients. Compared to the North American and Italian patients the DQ2 allele was also less prominent with 42 (41%) of patients.

For patients with SLA/LP positive AIH, HLA association had previously not been reported. Thus, we assessed the association of HLA of 29 patients with SLA positive, type 3 AIH (Table 3). Given a significantly smaller collective, there seemed to be a trend for SLA/LP positive AIH to be associated with the same HLA as AIH type 1. The most prevalent HLA in our patients with AIH were A2 in 16 patients (55%), B8 in 15 patients (52%), C7 in 22 patients (76%), DR3 in 12 patients (41%), and DQ2 in 13 patients (45%). Interestingly, only 3 patients did not carry either DR3 or DR4.

DISCUSSION

The etiology of AIH is still unknown. However, an underlying genetic predisposition has been suggested due to the fact that patients are predominantly of female gender and the association of the disease with certain HLA. Muratori *et al*^[4] recently published an extensive study on two large populations, Italian and North American, and demonstrated a distinct genetic association of HLA antigens with the disease. Since this study not only investigated the commonly studied HLA antigens type II but also extensively investigated the HLA antigens type I, this study was of significant value. In order to further elucidate HLA association with AIH and to compare our collective of patients with AIH, which is among the largest reported, to published populations in different regions of the world, we here present our data on HLA association in German patients.

Within the Italian population significant association of AIH was demonstrated for HLA antigens B8 (32%), C7 (51%), DQ2 (53%) and for the combined HLA type B8-DR3-DQ2 (30%). The frequencies of B8 and C7 were confirmed by our study (45% and 63%). In addition, an earlier British study had also reported on an association of increased frequency of C7 with AIH type 1^[6]. In contrast, DQ2 was found less prominent in German patients compared to Italian (41% *vs* 53%) and North American

Table 2 Prevalence of HLA subtypes in German patients with autoimmune hepatitis type 1 and comparison to worldwide populations

HLA	Germany <i>n</i> = 142	Italy ⁵ <i>n</i> = 57	N. America ⁵ <i>n</i> = 149	N. America II ⁸ <i>n</i> = 161	UK ⁶ <i>n</i> = 87	West India ¹⁰ <i>n</i> = 20	Japan ⁷ <i>n</i> = 77	China ⁹ <i>n</i> = 32	Brazil ⁸ <i>n</i> = 115	Turkey ¹¹ <i>n</i> = 17
A2	48%					16%				
B8	45%	32%	49%			3%				
C7	63%	51%			70%	29%				
DR3	38%	30%	52%	47%		11%	31%	16%	32%	17%
DR4	38%	23%	43%	45%		3%	83%	50%	16%	59%
DR7	16%	16%	15%							
DR11	13%	18%	7%							
DR13	13%	26%	16%							
DQ2	41%	53%	57%							
B8-DR3-DQ2	27%	30%	48%							

(41% *vs* 57%) patients, but still at a higher frequency compared to the Italian control population (30%). Finally, the combined HLA subtype B8-DR3-DQ2 was less frequent in German patients, especially compared to North American patients (27% *vs* 48%). This may mostly be due to the significant lower frequency of the DQ2 HLA in German patients.

On the contrary, our patients displayed DR4 HLA frequency that was highly similar to the North American patients and significantly higher than the reported Italian HLA associations. An association of DR4 with AIH type 1 had been reported and in other populations, especially in Japan (83%) and Brazil (50%) it had an even higher frequency^[7,8].

Frequency of HLA DR3 was highly similar between German and North American patients (45% and 52%), which were clearly higher than in all other populations reported thus far. Comparable to Italian patients DR3 was found at 31% in Japanese and 32% in Brazilian patients. In smaller populations from Western India, China and Turkey, this HLA antigen was even found at 11% to 17%^[9-11]. Moreover, 49 of all patients carried neither DR3 nor DR4. However, a search for highly prevalent HLA in these patients did not reveal any additional, obvious new association, independent of DR3 or DR4.

All other HLA frequencies, specifically reported and compared by Muratori were comparable between our collective and the Italian and the North American study groups. The results of all three study groups are summarized in Table 2.

To date, most studies on HLA association with AIH mainly focused on AIH type 1 patients and reported only a few AIH type 2 patients. Thus far, HLA association of SLA/LP positive AIH has not been reported in a significant number of patients. Therefore, we extended our analysis of HLA antigen association to SLA/LP positive AIH. Given a smaller group of only 29 patients, HLA frequencies seemed to be similar to patients with AIH type 1. Comparable to AIH type 1, a high frequency of C7 (77%) was found. Frequencies for A2, B8, and DR3 were comparable to those of our German patients with AIH type 1 but also to AIH type 1 patients of the Italian and North American groups, who had not reported data on their AIH type 3 patients. Clearly a higher frequency of

Table 3 Most prevalent HLA subtypes in German patients with SLA/LP positive autoimmune hepatitis

HLA	AIH SLA/LP positive German patients <i>n</i> = 29
A2	16 (55%)
B8	15 (52%)
C7	22 (76%)
DR3	12 (41%)
DQ2	13 (45%)

HLA DQ2 was observed in patients with SLA/LP positive AIH compared to our patients with AIH type 1. However, these higher HLA DQ2 frequencies are comparable to the DQ2 frequencies of the Italian and North American groups. Together, these data suggest a common genetic association and background for AIH types 1 and 3. This is in accordance with the observation that type 1 and SLA/LP positive AIH are also comparable with respect to their clinical course^[2].

In conclusion, German AIH type 1 patients were demonstrated to be genetically distinct from Italian or North American patients and other populations, especially with respect to a significantly lower frequency of HLA DQ2 and a lower occurrence of HLA B8-DR3-DQ2. Other HLAs were found at similar frequencies, suggesting an underlying genetic background of AIH type 1. Analysis of a smaller group of 29 patients with SLA/LP positive AIH pointed towards comparable HLA frequencies in patients with AIH type 1 and SLA/LP positive AIH, which is in accordance with a similar clinical course. The challenge is yet to investigate whether these findings may help to better understand the etiology of AIH, to predict prognosis of the disease, or to further improve therapeutic concepts.

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Expression pattern of leptin and leptin receptor (OB-R) in human gastric cancer

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Abstract

AIM: To examine the expression of leptin and its receptor, OB-R, in normal gastric mucosa and neoplasia.

METHODS: By immunohistochemical staining using specific antibodies, we evaluated the expression of leptin and OB-R in 207 gastric carcinomas (100 early and 107 advanced carcinomas) and analyzed their relationship with clinicopathological features.

RESULTS: Both normal gastric epithelium and carcinoma cells expressed a significant level of leptin. In cases with OB-R staining, carcinoma cells showed OB-R-positive expression, but the intensity was weaker than that in normal mucosa. The expression of OB-R showed a significant correlation with the level of leptin expression. The expression levels of both leptin and OB-R tended to increase as the depth of tumor invasion or TMN stage increased ($P < 0.01$). Lymph node metastasis was detected in 49.5% (47/95) of leptin-strong cases and in 50.5% (48/95) of OB-R-positive cases, and the rate was 33% (37/112) in leptin-weak cases and 17% (19/112) in OB-R-negative cases. Both venous and lymphatic invasion also tended to be observed frequently in positive tumors as compared with negative tumors. Interestingly, in the 96 leptin- or OB-R-positive tumors, hematogenous metastasis was detected preoperatively in 3 (3.1%) patients. In contrast, none of the carcinomas that lacked expression of leptin and OB-R showed hematogenous metastasis.

CONCLUSION: Overexpression of leptin and expression of OB-R may play a positive role in the process of progression in gastric cancer. Functional upregulation of leptin/OB-R may have a positive role in the development and initial phase of progression in gastric cancer.

INTRODUCTION

Previous studies have shown a positive association between adiposity and increased risk of cancers of the endometrium, kidney, gallbladder (in women), breast (in postmenopausal women), and colon (particularly in men)^[1-9]. Consistently, a recent large scale cohort study demonstrated that increased body weight is associated with increased mortality for all cancers combined, as well as for cancers at various specific sites^[10].

The adipocyte-derived cytokine, leptin, is thought to play a key role in the control of satiety, energy expenditure, and various reproductive processes^[11-13]. Leptin is a peptide hormone composed of 167 amino acids with a molecular mass of 16 kDa^[14]. Generally, plasma leptin level is representative of body fat mass^[15-18] and increases in a logarithmic fashion with an increase in body mass in mice^[19]. Leptin controls body mass and metabolism by affecting the metabolic, neuroendocrine, reproductive and hematopoietic systems^[20]. In cancer, there is regulatory dysfunction in metabolic, neuroendocrine and other systems. Although initially thought to be exclusively expressed in and secreted by adipocytes, recent studies have shown that leptin expression can be detected in a number of additional tissues, including the placenta^[21], gastric mucosa^[22], pituitary cells^[23] and hepatic stellate cells^[24]. More importantly, leptin has been shown to regulate cell proliferation in diverse normal and malignant tissues and to stimulate the proliferation of certain normal hematopoietic and epithelial cells. Leptin has also been shown to promote the invasiveness of premalignant colon and kidney epithelial cells *in vitro*^[25]. These findings suggest the possibility that leptin may be critically involved in the promotion of cancer.

Leptin exerts its action through the leptin receptor (OB-R), a member of the cytokine family of receptors, which was also detected in both rat and human gastric mucosa. Further studies, however, have shown that leptin receptors are expressed in many other tissues, including

the brain, placenta, pancreas, adrenal gland, hematopoietic cells, liver, lung and heart^[21,24,26-28]. In addition, OB-R has been identified in malignant cells, including lung and gastric carcinoma and leukemic cells^[22,29-32].

In this study, therefore, we used antibodies to leptin and OB-R, and immunohistochemically characterized the expression pattern of these two proteins in gastric carcinoma and evaluated the possible role of leptin in the tumorigenesis and spread of gastric cancer.

MATERIALS AND METHODS

Two hundred and seven carcinomas, which were surgically resected in the Department of Surgery, The University of Tokyo, from 1991 to 2002, were included in this study. In all cases, serial-step sections 3-mm wide were cut, fixed in 10% formalin solution, and then embedded in paraffin. All the resected primary tumors and regional lymph nodes were histologically examined by hematoxylin-eosin staining according to the Japanese Classification of Gastric Carcinoma^[33]. Tumors were histologically classified into two types based on the predominant features: differentiated type (well and moderately differentiated adenocarcinoma) and undifferentiated type (poorly differentiated adenocarcinoma and signet ring cell carcinoma). In addition, we examined several discrete histological parameters, including lymphatic invasion, venous invasion and lymph node metastasis.

Immunohistochemical study of leptin and OB-R

We investigated the expression of OB-R and leptin with immunohistochemical staining using affinity purified rabbit polyclonal antibodies against leptin (Santa Cruz, Biotech, CA, USA) and goat polyclonal antibodies against OB-R (M-18, Santa Cruz Biotech)^[30,32], respectively. Sections (3- μ m thick) were deparaffinized in xylene, hydrated through a graded series of ethanol, and heated in a microwave oven for two 7-min cycles (500 W). After being rinsed in phosphate buffered saline (PBS), endogenous peroxidase activity was inhibited by incubation with 0.3% hydrogen peroxide in 100% methanol for 30 min. After 3 washes in PBS, nonspecific reaction was blocked by incubation with PBS containing 5% skimmed milk for 30 min at room temperature, and then the sections were incubated with normal rabbit or goat serum for 30 min. The sections were incubated overnight at 4°C in humid chambers with the primary antibody to leptin at 1/70 dilution or the primary antibody to OB-R at 1/100. After three washes with PBS, the sections were incubated with biotinylated rabbit anti-goat or anti-rabbit immunoglobulin for 30 min. After washing again with PBS, the slides were treated with peroxidase-conjugated streptavidin for 30 min, and developed by immersion in 0.01% H₂O₂ and 0.05% diaminobenzidine tetrahydrochloride for 3 min. Light counterstaining with Mayer's hematoxylin was performed.

Statistical analysis

All statistical calculations were carried out using Stat View-J 5.0 statistical software (SAS Institute, USA). Student's t-test and Wilcoxon's test were used to analyze data.

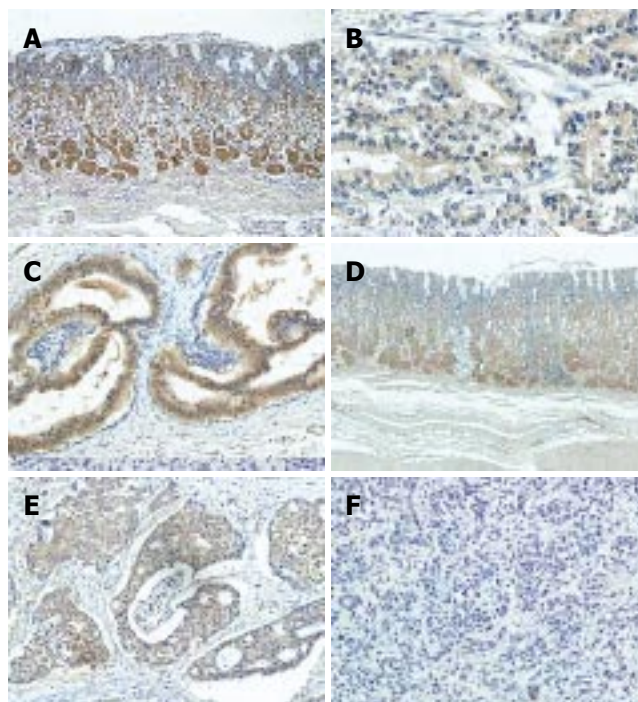


Figure 1 Immunohistochemical staining of leptin and OB-R in normal gastric mucosa and cancer. **A:** Leptin in normal mucosa; **B:** Leptin weak type in gastric cancer; **C:** leptin strong type in gastric cancer; **D:** OB-R in normal mucosa; **E:** OB-R positive type in gastric cancer; **F:** OB-R negative type in gastric cancer.

Differences with a *P* value of less than 0.05 were considered to be statistically significant.

RESULTS

Immunohistochemical detection of leptin and OB-R in normal mucosa and carcinoma

In all cases, the lower part of the fundic glands in the normal part of the mucosa expressed a significant level of leptin, suggesting that leptin is mainly produced in chief and parietal cells (Figure 1A). Leptin could be detected in the cytoplasm as well as the cell membrane, but not in the nucleus. However, the surface epithelium of normal gastric mucosa totally lacked expression of leptin. This staining pattern was similar to that described in the gastric epithelium in a previous report^[22].

Gastric carcinoma cells mostly showed positive immunoreactivity, although the staining intensity varied among the samples. According to the staining pattern, tumors were subdivided into two groups. When investigators agreed that the staining intensity of carcinoma cells was significantly weaker than that of chief and parietal cells in corresponding normal mucosa, those tumors were categorized as having weak expression (Figure 1B). In contrast, when carcinoma cells stained to a similar degree or more strongly than normal gastric mucosa, those tumors were categorized as having strong expression (Figure 1C).

OB-R was also detected in normal mucosa, and the immunostaining pattern was mostly consistent with that of leptin staining (Figure 1D). In cancer tissue, however, some carcinoma cells showed significant expression while

Table 1 Relationship between expression of Ob-R and leptin

	Ob-R expression		<i>P</i>
	Positive (67)	Negative (140)	
Leptin expression			
Strong (74)	45	29	< 0.001
Weak (133)	22	111	

others were mostly negative for OB-R. In tumors positive for OB-R, most of the carcinoma cells were equally stained and heterogeneity was rarely observed in each sample (Figure 1E), while in negative tumors a few carcinoma cells were stained only faintly (Figure 1F).

The relationship between the expression patterns of leptin and OB-R is presented in Table 1. Among 74 carcinomas with strong expression of leptin, 45 (60.8%) also expressed OB-R strongly, while only 29 carcinomas (39.2%) lacked expression of OB-R. In the 133 carcinomas with weak leptin expression, 111 (79.3%) also lacked expression of leptin OB-R. Hence, the expression of leptin and OB-R was significantly correlated in gastric cancer ($P < 0.001$).

Clinicopathological features and relation to expression of leptin and OB-R

Table 2 shows the correlation between leptin and OB-R expression and clinicopathological data in the 207 carcinomas cases. No significant difference was observed in age, preoperative tumor markers or tumor size between the positive and negative groups. Interestingly, positive expression of leptin was found in 64 of 155 male patients (41.3%) versus 10 of 52 female patients (19.2%), and the difference was statistically significant ($P < 0.05$). The relationship of BMI with leptin expression in the two groups did not show a significant association. The stomach is anatomically divided into three portions; upper (U), middle (M), lower (L) parts^[33]. Interestingly, the percentage of tumors with strong expression of leptin was higher in those located in the upper part (23/51; 45.1%) than in those in the middle (36/94; 38.3%) and lower (15/62; 24.2%) parts, and this was statistically significant ($P < 0.05$).

The expression levels of both leptin and OB-R increased as the depth of tumor invasion or TMN stage increased ($P < 0.01$). When leptin expression was compared with histological type, 45 of 88 (51.1%) well differentiated carcinomas expressed leptin strongly, while 90 of 119 (75.6%) undifferentiated carcinomas showed weak staining ($P < 0.001$). Moreover, the percentage of tumors with OB-R-positive expression was significantly higher in differentiated carcinomas (48.9%) than in undifferentiated carcinomas (20.1%) ($P < 0.001$). Thus, both leptin and OB-R were expressed at a higher level in differentiated carcinomas as compared with undifferentiated carcinomas ($P < 0.001$).

Venous as well as lymphatic invasion was more frequently observed in tumors with high leptin and positive OB-R expression. Accordingly, lymph node metastasis was detected in 71.6% (48/67) of OB-R-positive cases, which

was significantly higher than 31.3% (47/150) of OB-R-negative cases. Interestingly, in the 74 tumors with high leptin expression, hematogenous metastasis was detected preoperatively in 3 (4.1%) patients, and peritoneal dissemination was detected intraoperatively in 5 (6.8%) patients. However, in 133 tumors with low leptin expression, only one case showed peritoneal dissemination and none was associated with hematogenous metastasis ($P < 0.05$).

DISCUSSION

Leptin is well known to play a major role in the regulation of weight and adiposity. Recently, many studies have shown that increased body weight is associated with increased risk of cancer and cancer-related mortality, suggesting a possible role of leptin in the pathogenesis of cancer. Leptin is reported to be abundantly produced in the stomach^[34,35]. In gastric carcinoma, some reports have shown that obesity is one of the main risk factors^[36-42]. These findings suggest that leptin may be critically involved in the development and progression of gastric cancer. This idea encouraged us to evaluate the expression of leptin and its receptor in gastric cancer tissues.

In our series, leptin and OB-R were predominantly expressed in chief and parietal cells but not in the surface epithelium in normal parts of the gastric mucosa that were adjacent to cancer tissue, which is mostly consistent with data of previous studies^[22,43,44]. However, carcinoma cells showed a variety of staining patterns for leptin or OB-R. Leptin was detected in all carcinoma cells, although the level of expression could be divided into two categories according to the staining intensity, whereas OB-R was detected in some tumors but not in others, and the level of expression of leptin showed a positive correlation with OB-R expression. This suggests the existence of a common regulatory mechanism in the expression of leptin and its receptor in the gastric epithelium.

The main finding in our study was that the expression levels of both leptin and OB-R tended to increase as the depth of tumor invasion or TMN stage increased ($P < 0.01$). Moreover, nodal and distant metastasis, as well as pathological lymphatic or vascular invasion, was frequently detected in leptin-strong and OB-R-positive tumors as compared with leptin-weak and OB-R-negative tumors. Shuneider *et al.*^[43] reported that leptin led to significantly increased proliferation in AGS cells, and the MAP-kinase-1 specific inhibitor U0126 blocked leptin-induced cell proliferation in a dose-dependent manner. Tessitore reported that in colorectal cancer patients, plasma leptin level in stage IV patients was significantly higher than that in stage I patients. In addition to stimulating proliferation, leptin has been shown to promote invasiveness of renal and colonic epithelial cells via PI3-kinase-, rho- and rac-dependent cascades^[25]. All these findings support that leptin may have a promoting effect on cancer invasion and metastasis. Our findings were consistent with these results and suggest that leptin and OB-R might function as an autocrine growth factor during the development and progression of gastric cancer.

Another interesting finding was that the expression of

Table 2 Expression of leptin and clinicopathologic characteristics of patients

	Leptin expression			OB-R expression		
	High (74)	Low (133)	P	Positive (67)	Negative (140)	P
Age (yr)	62.7 ± 8.9	61.3 ± 11.2	0.98	64.2 ± 9.9	61.6 ± 10.8	0.167
Sex						
Male	64	91		53	102	
Female	10	42	0.003	14	38	0.33
BMI	22.4 ± 3.1	22.7 ± 2.8	0.48	22.4 ± 3.2	22.7 ± 2.8	0.49
Tumor markers						
CEA	8.6 ± 17.7	14.7 ± 111.3	0.65	9.9 ± 19.6	13.8 ± 108.3	0.79
CA19-9	89.8 ± 453.5	75.4 ± 347.6	0.81	138.3 ± 561.4	53.8 ± 270.8	0.16
Size (cm)	6.0 ± 3.2	5.1 ± 3.6	0.13	5.8 ± 3.2	5.2 ± 3.6	0.25
Location						
Upper part (51)	23	28		19	32	
Middle part (94)	36	58		29	65	
Lower part (62)	15	47	0.03	19	43	0.51
Depth of tumor invasion						
T1	24	76		20	80	
T2	28	29		27	37	
T3	18	28		16	30	
T4	4	0	0.001	4	0	< 0.001
Macroscopic type						
Elevated	57	62		48	71	
Depressed/flat	17	71	< 0.001	19	69	0.005
Histological type						
Differentiated	45	43		43	45	
Undifferentiated	29	90	< 0.001	24	95	< 0.001
TNM stage						
IA	20	67		15	72	
IB	17	23		15	25	
II	8	12		9	11	
III A	10	17		11	16	
III B	5	7		7	5	
IV	14	7	0.001	10	11	< 0.001
Lymphatic invasion						
Positive	39	45		39	46	
Negative	34	87	0.002	28	94	< 0.001
Venous invasion						
Positive	46	49		45	50	
Negative	28	84	< 0.001	22	90	< 0.001
Lymph node metastasis						
Positive	47	48		48	47	
Negative	37	85	0.052	19	93	0.001
Hematogenous metastasis						
Positive	3	0		2	1	
Negative	71	134	0.02	65	139	0.19
Peritoneal dissemination						
Positive	5	1		2	4	
Negative	69	133	0.01	65	136	0.95

leptin/OB-R was correlated with the differentiation of gastric cancer. In our series, cancers of undifferentiated type tended to have weak expression of leptin as well as negative OB-R expression as compared with differentiated cancers. In each type, expression of leptin/OB-R showed a positive association with stage and metastasis (data not shown). This suggests that the different expression

of leptin/OB-R was determined at the early stage of carcinogenesis. The carcinogenic pathway of differentiated type carcinoma is considered to begin with *H pylori* infection, followed by atrophic gastritis and intestinal metaplasia, and inappropriate activation of gut specific transcription factor CDX2 has an important role in the early stage of carcinogenesis. In contrast, dysfunction

of E-cadherin is considered to have critical roles in the development of undifferentiated carcinoma. The molecular interaction between leptin/OB-R and CDX2 or E-cadherin is an interesting subject for future research^[45].

In conclusion, we confirmed that the expression level of leptin/OB-R showed a positive correlation with the depth of tumor invasion, stage, and metastasis as well as tumor differentiation. Our findings suggest that coexpression of leptin and OB-R may have a positive role in the progression in gastric cancer in an autocrine or paracrine manner. Functional inhibition of leptin/OB-R might effectively suppress the growth and metastasis of gastric cancer.

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Luminal oxidants selectively modulate electrogenic ion transport in rat colon

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Abstract

AIM: To investigate the effects of luminal exposure to H_2O_2 and two related thiol oxidizing agents on basal and stimulated chloride secretion in native colon using electrophysiological and pharmacological approaches.

METHODS: Unstripped rat distal colon segments were mounted in Ussing chambers. Potential difference, calculated resistance and short-circuit current across unstripped colon segments were monitored with a dual voltage/current clamp. Paracellular permeability was assessed by measuring the mucosa-to-serosa flux of a fluorescent probe (FITC).

RESULTS: Luminal exposure to hydrogen peroxide transiently stimulated chloride secretion without altering barrier function. This stimulatory effect could be blocked by basolateral atropine but not indomethacin. The cysteine and methionine oxidizing compounds, phenylarsine oxide and chloramine T respectively, mimicked the effect of H_2O_2 , except for a drop in transcolonic resistance after 30 min. In contrast to the observed stimulatory effect on basal secretion, cAMP-stimulated electrogenic ion transport was blunted by luminal H_2O_2 . However, the Ca^{2+} -activated response remained unchanged.

CONCLUSION: H_2O_2 may be an important selective modulator of intestinal ion and water secretion in certain pathologic conditions such as inflammation or ischemia-reperfusion by multiple mechanisms.

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Key words: Chloride secretion; Ion transport; Rat distal colon; Hydrogen peroxide; Acetylcholine; Atropine

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INTRODUCTION

The colonic lumen is an extraordinarily aggressive milieu containing high concentrations of oxidizing compounds^[1] as a result of the digestion of nutrients, the presence of dietary oxidants and the metabolic activity of resident colonic flora. Moreover, many gastrointestinal diseases, such as intestinal ischemia, ischemic colitis or inflammatory bowel disease, are associated with an increase in the concentration of both reactive oxygen and nitrogen species in the intestinal mucosa^[2], generated by either activated neutrophils infiltrating the colonic crypts or epithelial cells submitted to ischemia/reperfusion. Therefore, colonic epithelial cells and submucosal tissue are under continuous luminal oxidative stress in states of health and disease, but little is known about the impact of luminal oxidants on mucosal functions in intact native mammalian colon since most studies have been carried out in cell lines.

Oxidants have been shown to interfere with epithelial ion transport and barrier function. In intestinal model cell lines, addition of hydrogen peroxide, either basolaterally or apically, first activates vectorial Cl^- transport and then inhibits cAMP-activated anion secretion. The inhibitory effect on the forskolin-stimulated response appears to be the result of the blockade of apical Cl^- conductance and the inhibition of $Na^+-K^+-ATPase$ activity^[3]. In animal models, serosal application of H_2O_2 , at micromolar concentrations, to muscle-stripped rat colon evokes a transient increase in anion secretion^[4]. Yet, the role of luminal oxidants on stimulated transport in intact native colon remains poorly understood. Therefore, our aim was to investigate the effect of luminal H_2O_2 on basal and stimulated electrogenic ion transport in rat distal colon.

MATERIALS AND METHODS

Rat distal colon model in vitro

Male Sprague-Dawley rats weighing 250-350 g were killed by intraperitoneal administration of sodium pentobarbital.

Animals were handled in accordance with the protocols approved by Comisión de Investigación-Hospital Clínico San Carlos, in compliance with both Spain's and European Union's regulations for the use of animals in biomedical research.

The method to obtain samples for electrophysiological studies has been reported elsewhere^[5,6]. In brief, a 5-cm segment of distal colon was surgically removed and placed in iced buffer solution. The specimen was opened along its mesenteric side. Unstripped colonic segments were mounted in modified chambers (Costar, San Diego CA) with a surface area of 0.64 cm². These chambers could provide individualized access to both the luminal and the serosal compartments of the colon. Both chambers were bathed with an identical volume (5 mL) of the buffer solution containing 122.0 mmol/L NaCl, 2.0 mmol/L CaCl₂, 1.3 mmol/L MgSO₄, 5.0 mmol/L KCl, 20.0 mmol/L glucose and 25.0 mmol/L NaHCO₃ (pH 7.4 when gassed with 950 mL/L of O₂ and 50 mL/L CO₂ at 37°C).

Electrophysiology

Two Ag/AgCl electrodes connected to a dual voltage-current clamp (World Precision Instruments ECV 4000, Sarasota-FL, USA) were placed in the apical and basolateral chambers. Spontaneous transepithelial potential difference (E₀; lumen negative, in mV) could therefore be monitored. Potential difference values were corrected for the junction potentials (< 0.1 mV) between the luminal and the serosal solutions. Two additional electrodes were used to apply a 50 µA current through the mounted colon. The resulting potential difference was measured (E₅₀). Transcolonic resistance (TR, in Ω·cm²) was calculated with E₀ and E₅₀ values by Ohm's law, reflecting epithelial viability and intestinal barrier function. Short circuit current (I_{sc}, in µA/cm²) could be obtained with E₀ and TR values by Ohm's law, indicating the amount of electrical current needed to nullify the spontaneous potential difference between the apical and the basolateral surfaces.

Electrophysiological experiments were carried out after the samples were mounted in the modified chambers and bathed in buffer solution until stable electrical activity was reached. The response of rat colon exposed to luminal oxidants to the muscarinic blocker (atropine) was studied after it was added to the basolateral chamber in the continued presence of both compounds. Similarly, the effect of two different secretagogues (forskolin and carbachol) on ion transport was assessed following the same protocol (luminal incubation + continued presence of the oxidant).

Paracellular permeability

Paracellular permeability was assessed by measuring the mucosal-to-serosal flux of fluorescein isothiocyanate (FITC; MW: 376.3) as previously described^[6]. Paired rat distal colon segments were mounted in chambers and bilaterally incubated in regular buffer for 10 min for equilibration. Subsequently, the apical buffer was replaced with a FITC-containing solution (140 µmol/L) with or without hydrogen peroxide. Basolateral buffer aliquots were collected at 0, 5, 15, 30 and 60 min after apical buffer replacement and the

fluorescent emission at 520 nm after excitation at 480 nm was measured with a spectrofluorometer (Bio-Tek FL600 Fluorescence Microplate Reader, Bio-Tek Instruments GmbH, Bad Friedrichshall, Germany). A calibration curve (fluorescence vs FITC concentration) was generated to calculate the concentration of FITC in the serosal chamber. The apparent permeability coefficient (P_{app}) was calculated according to the equation $P_{app} (cm/seg) = (dI_2/dI_1) \cdot V \cdot A^{-1} \cdot C_0^{-1}$, where (dI_2/dI_1) is the net increase in FITC concentration in the serosal buffer in a given interval of time (seconds), V is the volume (in milliliters) of the basolateral compartment, A is the surface of the colon segment (in square centimeters) and C_0 is the concentration in the apical chamber.

Materials

Cyclic AMP and Ca²⁺-dependent secretion was stimulated with forskolin (10 µmol/L) and carbachol (100 µmol/L), respectively. Phenylarsine oxide (PAO, 0.2 mmol/L) and chloramine T (CIT, 5 mmol/L) were used as non-physiologic thiol oxidants. For muscarinic receptor inhibition, atropine at a concentration of 1 mg/L was employed. Indomethacin (1-10 µmol/L) was used for blockage of cyclo-oxygenase activity. Forskolin and indomethacin were added to both chambers (luminal and serosal). However, exposition to the muscarinic agonist and its antagonist was only from the serosal side. All the compounds were obtained from Sigma, Madrid-Spain.

Statistical analysis

Results were presented as mean ± SD. Student's *t* and repeated measure ANOVA tests were used for statistical comparisons when indicated. *P* < 0.05 was considered statistically significant.

RESULTS

Luminal exposure to H₂O₂ (up to 10 mmol/L) for 30 min did not alter either transcolonic resistance (TR of H₂O₂-treated samples = 92.2% ± 11.7% of controls; *n* = 6 for each group; NS) or paracellular permeability (P_{app} = 1.24 × 10⁻⁶ ± 0.16 × 10⁻⁶ for control vs 0.95 × 10⁻⁶ ± 0.35 × 10⁻⁶ for 10 mmol/L H₂O₂; *n* = 6 for each group, NS). However, as shown in Figure 1, a transient increase in I_{sc} occurred 5 min after the addition of the oxidant to the apical chamber (peak I_{sc} = 46.7 ± 2.8 µA/cm² for control samples, *n* = 5; vs 85.9 ± 9.6 µA/cm² for 10 mmol/L H₂O₂; *n* = 6, *P* < 0.01). This secretory response was dose-dependent (from 100 µmol/L to 10 mmol/L) and peaked at a concentration of 8 mmol/L (ΔI_{sc} = 32.3 ± 3.4 µA/cm² at 8 mmol/L; *n* = 4 for all groups; *P* < 0.05 for the comparison between controls and oxidant-treated samples). However, it was not specific for H₂O₂ since two widely used cysteine and methionine oxidizing agents, phenylarsine and chloramine T, at concentrations used by other investigators to study epithelial barrier function in cell lines (0.2 mmol/L and 5 mmol/L respectively), generated the same secretory response after 5 min but induced a slight drop in transcolonic resistance 30 min after addition (Table 1).

In order to investigate the implication of different

Table 1 Transcolonic resistance and maximal short circuit current in rat distal colon exposed to phenylarsine oxide (PAO, 0.2 mmol/L) and chloramine T (CIT, 5 mmol/L) for 30 min

Group	n	TR ¹ at 30 min	peak Isc ²
Control	8	113 ± 8.0	66.4 ± 6.8
PAO (0.2 mmol/L)	4	77 ± 14.0 ^a	131.6 ± 16.7 ^a
CIT (5 mmol/L)	4	66.5 ± 4.3 ^a	166.6 ± 27.8 ^a

^aP < 0.05 vs control; ¹Transcolonic resistance; ²Short circuit current.

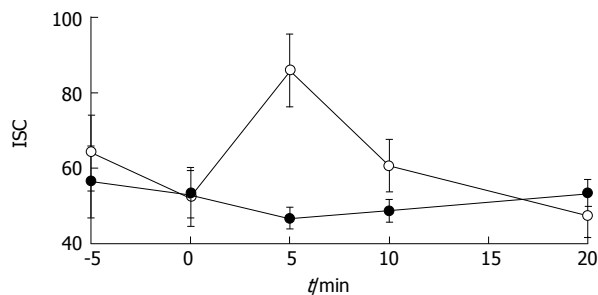


Figure 1 Luminal H₂O₂ transiently stimulates Cl secretion. After the addition of 10 mmol/L H₂O₂ to the apical surface of unstripped rat distal colon segments at time 0 (in white circles, n = 4), basal Isc increased significantly compared to control (black circles, n = 4), with maximal secretion occurring 5 min after the addition of the oxidant (P < 0.001).

secretagogues in H₂O₂-induced secretion, colonic samples were first incubated with varying concentrations of indomethacin (1–10 μmol/L) and subsequently exposed to the three oxidizing compounds. No inhibition of the Isc rise caused by 10 mmol/L H₂O₂ was observed. For example, samples incubated with 2 μmol/L indomethacin after addition of 10 mmol/L H₂O₂ yielded a peak Isc of 110 ± 13.7 μA/cm², whereas for controls the peak Isc was 134 ± 14.2 μA/cm² (n = 9 for each group; NS). The same result was observed with the other two oxidants (data not shown). Since other mediators, such as acetylcholine, might be involved in the observed effect, the response to luminal oxidants after muscarinic receptor blockade was investigated. Interestingly, the secretory response elicited by the three compounds was suppressed by basolateral preincubation with atropine (1 g/L). The ΔIsc in atropine-treated samples after exposure to 10 mmol/L H₂O₂ was -1.3 ± 8.6 μA/cm², whereas in samples incubated in regular buffer the ΔIsc in response to the same concentration of H₂O₂ was 55.2 ± 13.7 μA/cm² (n = 6, P < 0.01). Both PAO and chloramine T displayed a similar behaviour (Figures 2A and B).

Subsequently, the effect of luminal H₂O₂ on stimulated secretion was studied. After 30 min of incubation with 10 mmol/L H₂O₂, when no drop in permeability occurred (TR after incubation for 30 min = 116.6 ± 15.8 Ω·cm² H₂O₂-treated samples vs 87.8 ± 19.7 Ω·cm² for control; n = 6 and n = 5 respectively; NS), forskolin stimulated-secretion was inhibited (Figure 3) (maximal Isc = 69.3 ± 9.4 μA/cm² for H₂O₂-treated samples vs 134.1 ± 23.1 μA/cm² for control (n = 6 and n = 5 respectively; P < 0.01). This inhibitory action was dose-dependent with an IC₅₀-0.7 mmol/L. Since Ca²⁺-dependent agonists could activate

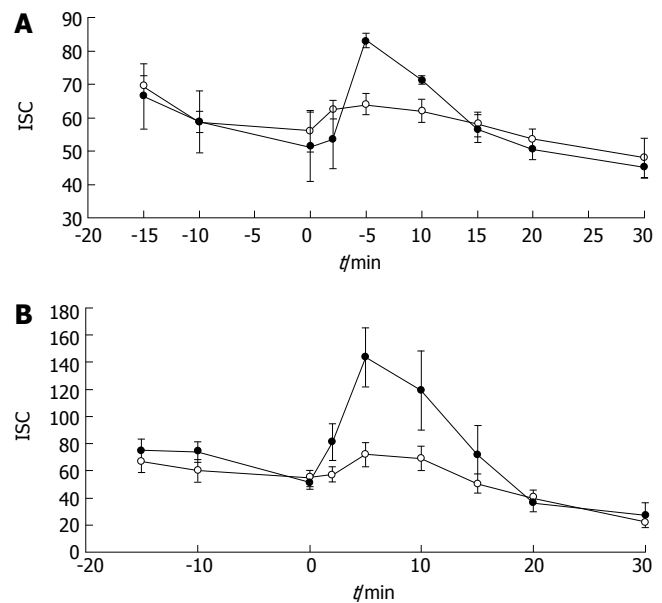


Figure 2 Oxidant-stimulated secretion is sensitive to basolateral atropine. Both phenylarsine oxide (PAO, 0.2 mmol/L) and chloramine T (CIT, 5 mmol/L) showed a similar prosecretory effect when they were added luminally to rat distal colon. **A:** Preincubation of colon segments with basolateral atropine (1 g/L) for ten minutes (added at time -10; in white circles, n = 3) suppressed the increase in Isc induced by luminal cysteine oxidizing agent PAO added at time 0 (control in black circles; n = 3; P < 0.01 between groups); **B:** A similar result was observed with the methionine oxidizing compound chloramine T. Basolateral incubation with 1 g/L of atropine (added at time -10; in white circles, n = 8) suppressed the secretory response caused by CIT added at time 0 (in black circles, n = 8; P < 0.001 between groups).

distinct signaling pathways and membrane transporters to generate electrogenic ion transport, we studied the effect of 10 mmol/L H₂O₂ on the carbachol-stimulated secretory response. In this case, samples exposed to the oxidant luminally or to regular buffer responded to the Ca²⁺ agonist in the same fashion (Figure 4).

DISCUSSION

Even under normal conditions, colonic epithelial cells are continuously exposed to luminal oxidants from different sources^[7,8]. Since these compounds may interfere with epithelial functions, further understanding of the specific effects of reactive oxygen species on ion transport is necessary to design therapeutic strategies to prevent cell dysfunctions, such as hypersecretion and energy depletion, during inflammation or ischemia^[1,2]. In this study, we showed that luminal hydrogen peroxide, within a physiologically relevant concentration range, could transiently activate chloride secretion without altering either electrical resistance or paracellular permeability in rat distal colon. This effect might be due to either direct stimulation of membrane transport proteins or due to the activation of intracellular second messengers in epithelial cells. In fact, mucosal and serosal addition of H₂O₂ could potentiate Cl secretion in a synergistic fashion^[9] in T84 cells previously stimulated with cAMP-agonists, suggesting that the Ca²⁺ signaling pathway might be involved in H₂O₂-triggered secretory response.

However, the maximal Isc induced by H₂O₂ in the T84

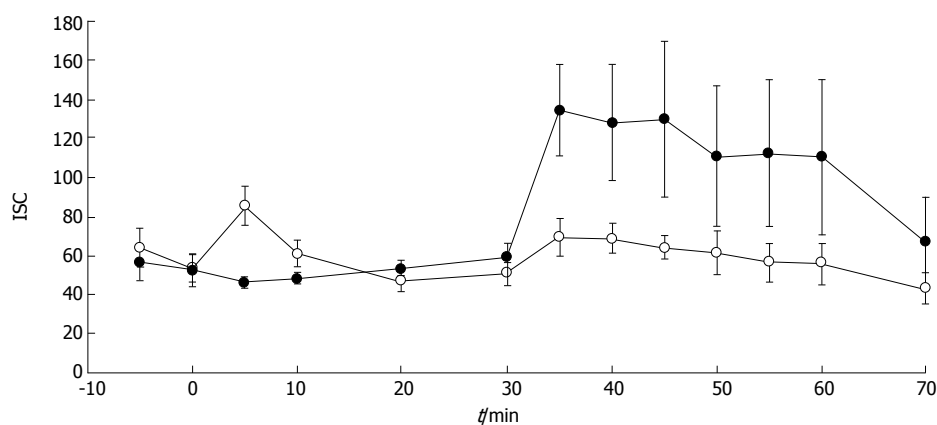


Figure 3 cAMP-activated secretion is blocked by luminal H_2O_2 . Incubation of rat distal colon with 10 mmol/L H_2O_2 for 30 min (white circles; $n = 6$) inhibited the rise in Isc caused by 10 $\mu\text{mol/L}$ forskolin added at time 0 (control in black circles; $n = 6$; $P < 0.01$) in white circles, $n = 8$) and suppressed the secretory response caused by CIT added at time 0 (in black circles, $n = 8$; $P < 0.001$ between groups).

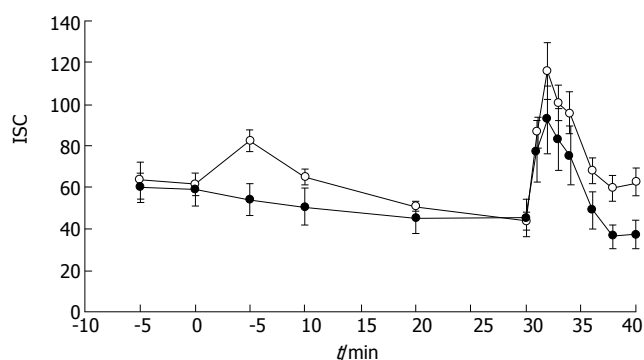


Figure 4 Ca^{2+} -stimulated Cl^- secretion is not affected by luminal H_2O_2 . Incubation of rat distal colon with 10 mmol/L H_2O_2 for 30 min (white circles; $n = 6$) slightly enhanced the response induced by 100 $\mu\text{mol/L}$ carbachol (control in black circles; $n = 6$; NS).

cell line is rather small compared to that observed in our model. Sugi *et al*^[10] suggested that oxidants might enhance the response to secretagogues by priming of transport proteins in epithelial cell membrane. Interestingly, our results suggest that sudden increases in the concentration of luminal oxidants could activate secretion by releasing acetylcholine rather than by directly stimulating epithelial cells, which is independent from the generation of prostaglandins. This assertion is supported by the fact that the rise in Isc induced by three different oxidants-hydrogen peroxide, phenylarsine oxide and chloramine T, is almost completely suppressed by incubation with basolateral atropine but not with indomethacin at several concentrations. These findings are in contrast with the observations reported by Karayalcin *et al*^[11], who found that basolateral hydrogen peroxide stimulates indomethacin-inhibitable secretion in stripped rat colon, which can be only partially blocked by atropine. Likewise, Tamai *et al*^[4] showed that H_2O_2 increases Isc probably by stimulating release of arachidonate metabolites and neurotransmitters. This divergent finding may be due, at least in part, to the different “*in vitro*” models used. We studied the unstripped rat distal colon^[12,13] because it could more accurately reflect the native tissue “*in vivo*” than the mucosa-submucosa preparations. Furthermore, Tamai *et al*^[4] and Karayalcin *et al*^[11] have used lower H_2O_2 concentrations and applied the oxidant to the serosal aspect of the stripped colonic wall.

An alternate explanation would imply that luminal

H_2O_2 exerts a distinct action on each signaling pathway. That is, it might be priming the Ca^{2+} -dependent pathway while blocking the cAMP-dependent pathway. Thus, the stimulatory action of prostaglandins on colonic epithelial cells would be abolished and therefore, would become “invisible”. To further support this hypothesis, previous studies in T84 cells have shown that incubation with H_2O_2 blocks chloride secretion stimulated by the cAMP agonist forskolin^[3]. Similarly, we found that H_2O_2 could inhibit the cAMP-dependent secretory response, but not the activation of electrogenic ion transport by carbachol. In fact, the response to the Ca^{2+} agonist was slightly augmented. This finding points at a cyclic nucleotide-dependent membrane transport protein as the target for luminal oxidants. From previous studies, one can speculate that apical cAMP-dependent Cl^- channels are the most likely sites of action for these compounds^[3,14]. The Na^+/K^+ -ATPase is another potential target for oxidizing agents^[3]. However, the discrepancy between the observed inhibition of the cAMP-dependent increase in vectorial anion transport and the absence of such an action on Ca^{2+} -stimulated secretion renders this explanation unlikely.

In conclusion, our results suggest that H_2O_2 may be an important selective modulator of intestinal ion and water secretion in certain pathologic conditions such as inflammation or ischemia-reperfusion by multiple mechanisms. Irrespective of the underlying mechanism of activation, the fact that an increase in the luminal concentration of reactive oxygen species triggers chloride secretion may be teleologically explained as a defensive mechanism for flushing microorganisms and toxic byproducts of bacterial metabolism away from the colonic lumen to dilute their concentration.

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RAPID COMMUNICATION

Timing of laparoscopic cholecystectomy for acute cholecystitis: A prospective non randomized study

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stectomy; Timing

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Abstract

AIM: To study the timing of laparoscopic cholecystectomy for patients with acute cholecystitis.

METHODS: Between January 2002 and December 2005, all American Society of Anesthesiologists classification (ASA) I, II and III patients with acute cholecystitis were treated laparoscopically during the urgent (index) admission. The patients were divided into three groups according to the timing of surgery: (1) within the first 3 d, (2) between 4 and 7 d and (3) beyond 7 d from the onset of symptoms. The impact of timing on the conversion rate, morbidity and postoperative hospital stay was studied.

RESULTS: One hundred and twenty-nine patients underwent laparoscopic cholecystectomy for acute cholecystitis during the index admission. Thirty six were assigned to group 1, 58 to group 2, and 35 to group 3. The conversion rate and morbidity for the whole cohort of patients were 4.6% and 10.8%, respectively. There was no significant difference in the conversion rate, morbidity and postoperative hospital stay between the three groups.

CONCLUSION: Laparoscopic cholecystectomy for acute cholecystitis during the index admission is safe, regardless of the time elapsed from the onset of symptoms. This policy can result in an overall shorter hospitalization.

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Key words: Acute cholecystitis; Laparoscopic cholecy-

INTRODUCTION

Laparoscopic cholecystectomy (LC) has been established as the treatment of choice for the management of acute cholecystitis (AC), despite initial reservations, regarding the impact of this policy on the conversion rate and morbidity^[1]. Several prospective randomized trials^[2-4] suggest the superiority of early (within 72 h) over the delayed (after a few weeks interval) intervention. This 72 h limit, however, is difficult to be kept in many cases for a variety of reasons, referring to both patients and physicians. On the other hand, there is a paucity of solid data as to what happens in the period after this 72 h time frame. The speculation of a worse outcome, when attempting LC for AC during the urgent admission beyond this very early phase, is experience rather than evidence-based.

In our daily practice, we have realized that only a small number of patients with AC are managed surgically within this "gold window" of 72 h from the onset of symptoms. If the remaining majority of patients with AC are managed conservatively with interval cholecystectomy to follow, then an increased total hospitalization and subsequently increased cost can be expected. Furthermore, the subgroup of patients who do not respond to conservative treatment, as well as those who relapse while awaiting an interval cholecystectomy should be considered^[5]. For these reasons, we have adopted a policy of performing a LC during the initial emergency/urgent admission for "all comers" with AC, regardless of time delay between its onset of symptoms and surgery.

In view of this policy, we examined prospectively the impact of the duration of symptoms on mortality, morbidity, conversion rate and postoperative hospital stay in patients who underwent LC for AC during the urgent (index) admission.

MATERIALS AND METHODS

Subjects

Between January 2002 and December 2005, all American Society of Anesthesiologists classification (ASA) I, II and III patients admitted or referred to our unit with AC under the care of one consultant surgeon (GT) with a special interest in HPB and laparoscopic surgery, were treated with LC during the index admission, regardless of the time elapsed from the onset of symptoms. ASA IV patients were usually offered ultrasound-guided percutaneous cholecystectomy and therefore, were excluded from the study. Patients were considered having AC when they had five out of the following six positive criteria: persistent right upper quadrant pain, temperature $> 37.5^{\circ}\text{C}$, WBC $> 10 \times 10^9/\text{L}$, positive Murphy's sign, presence of gallstones on ultrasound in combination with wall thickening and/or fluid at the gallbladder fossa. The diagnosis of AC was confirmed by intraoperative findings and pathologic specimens. Patients with strong evidence of concomitant common bile duct (CBD) stones were not excluded from the study, but were treated initially with preoperative endoscopic retrograde cholangiopancreatography (ERCP), sphincterotomy and CBD clearance, followed by LC after an interval of at least 24 h, in order to assure that no ERCP-related complication occurred. Patients with suspicion of CBD stones had preoperative MRCP, and if stones were detected, they were treated as above. Intraoperative cholangiogram was not performed in any of the cases. There were no other selection criteria and every effort was made to operate on all the patients as soon as theatre time was available, provided that any concomitant medical problem was previously dealt with. The latter resulted sometimes in what is called in the literature "physician delay"^[6]. Laparoscopic cholecystectomy was attempted in all cases under general anesthesia. The usual four-trocar technique was used (10 mm umbilical, 10 mm subxiphoid, 5 mm subcostal midclavicular line, 5 mm anterior axillary line) but additional trocar was used as necessary. The gallbladder was aspirated in most of the cases in order to be grasped, and dissection of the Calot's triangle structures was always performed close to the gallbladder wall. Retrograde dissection was only exceptionally performed, when in doubt about the triangle's structures after the initial dissection.

Methods

The patients were divided in three groups according to the time between onset of symptoms and operation: (1) within 3 d (early group), (2) between 4 to 7 d (intermediate group) and (3) ≥ 8 d (delayed group). All data including demographics, preoperative, operative findings and postoperative information were collected prospectively into a computerized database. The episode of AC was considered simple (oedematous, hydrops) or complicated (empyema, gangrenous, emphysematous, concomitant choledocholithiasis or pancreatitis). The aim of the study was to detect the impact of the time elapsed from onset of symptoms to operation on the conversion rate, 30-d mortality, 30-d morbidity with special attention to bile duct injury incidence and length of postoperative hospital stay.

Table 1 Impact of delay in laparoscopic cholecystectomy on outcomes

Outcome	I : ≤ 3 d (n = 36)	II : 4-7 d (n = 58)	III : ≥ 8 d (n = 35)	P
Conversion rate	1 (2.8%)	2 (3.4%)	3 (8.5%)	NS
Mortality	0	0	0	NS
Morbidity	3 (8.3%)	6 (10.3%)	5 (14.2%)	NS
Postop hospital stay	2 (1-6) d	2 (1-14) d	2 (1-35) d	NS

NS: no significant difference.

Statistical analysis

Statistical analysis was performed using the Arcus Quickstat biomedical statistical package (Research Solutions, UK) with the median values for continuous variables presented with range in parentheses. Fisher's exact test and Mann Whitney U test were used as appropriate to compare the groups to each other. $P < 0.05$ (two-tailed test) was considered statistically significant.

RESULTS

One hundred and twenty-nine patients underwent LC for AC during the index admission according to the protocol. During the same period some 453 elective laparoscopic cholecystectomies were performed by the same team. Thirty-six of the patients with acute cholecystitis (28%) had their operation within the first 3 d from the onset of their illness, 58 patients (45%) between 4 to 7 d and the other 35 patients (27%) after the first week. None of our patients had a more than 48 h delay due to unavailability of theatre space. Any other delay from the onset of symptoms to operation was attributed to either patients' delayed presentation/referral to our unit or to concomitant medical problems needing to be addressed preoperatively. Special mention should be made of a subgroup of patients with AC whose surgery was delayed due to the intake of anti-coagulants or more often anti-platelet agents, due to the dramatically increased use of these drugs during the last decade.

The impact of timing of LC on outcomes is shown in Table 1. Although the conversion rate was somewhat higher in the "delayed" group, this difference was not significant when this group was compared to either the "early" ($P = 0.35$, Fisher's exact test) or the "intermediate" group ($P = 0.36$, Fisher's exact test). Similarly, there was no significant difference in mortality, morbidity and postoperative hospital stay between the three groups of patients. Interestingly, this was noted despite the fact that a significantly higher number of complicated cases of AC were found among patients of the "intermediate" group, compared to those who underwent earlier operations. This was also reflected on the operative time difference between the groups (Table 2). No major bile duct injuries occurred. Four cases had bile leak, two in the "intermediate" group and two in the "delayed" group. The first was attributed to the gallbladder fossa, the second from an avulsed cystic duct and the remaining two from a friable cystic stump.

The first case eased spontaneously after 48 h, the other three cases with bile leak were treated successfully with ERCP, sphincterotomy and stenting of the common bile duct. Other complications and their treatment are shown in Table 3.

DISCUSSION

Acute cholecystitis which is generally found in approximately 20% of all admissions for gallstone disease^[7] is no longer considered a contraindication for laparoscopic cholecystectomy. In fact, urgent LC is now considered the optimal treatment of patients with AC^[1]. Early LC has been proven superior to delayed interval LC in most of the prospective randomized trials. It results in a shorter hospital stay and a shorter recuperation time while the conversion rate and morbidity remain similar with or even lower than delayed interval LC^[2-4]. How early is “early” is not clear in the literature, as this parameter has not been effectively tested in controlled randomized trials. All these prospective randomized trials comparing early and delayed interval LC, refer to the first 48-72 h for the early group, making this group somewhat highly selected. In daily practice very few patients are able to have surgical treatment during this short period of time, due to either patient or/and physician delay^[6]. Very often patients present with delay or they are referred with delay by their physicians. Others suffer from co-morbidities needing consultation with other specialties preoperatively, while some require other intervention preoperatively, i.e. ERCP. A significant number of patients take oral anti-coagulants or anti-platelet agents requiring reversing before surgery. For all these reasons many patients in reality cannot have surgery within this time frame. In the present series some 72% of patients with acute cholecystitis were treated surgically during the index admission beyond the 72 h boundary, which is not very different from the reported experience by other authors^[8-10]. There were no solid data regarding the optimal policy for this large group of patients treated outside this 72 h boundary. To our knowledge, there is only one small prospective randomized trial designed to address this issue^[11]. Chandler *et al*^[11] found that there is no difference in the conversion rate or morbidity between the early group (*n*: 21, surgery as soon as theatre schedule allowed) and the delayed group (*n*: 22, surgery during the index admission, after resolution of symptoms or failure to resolve on 5 d course of conservative treatment). Results from other comparative non-randomized trials of early and delayed LC during the urgent admission for AC are rather conflicting and most of these however indicate a higher conversion rate for the delayed group, but no difference in morbidity^[6,8,9,12-14]. The definition of the so called “early” group among trials is also confusing. Some trials define early group counting from the time of admission or diagnosis rather than the time of onset of symptoms. This could be sometimes misleading, as the onset time of episode could differ significantly from the time of admission. We believe that counting from the onset of symptoms is more representative of the reality. Furthermore, all the studies were designed by using a boundary either of 48, 72 or 96 h from either onset of symptoms or time of admission, in order

Table 2 Demographic and perioperative characteristics of patients with acute cholecystitis

Characteristic	I : ≤ 3 d (n = 36)	II : 4-7 d (n = 58)	III : ≥ 8 d (n = 35)
Male / Female	15/21	19/39	16/19
Age (median)	55 (19-76) yr	65 (24-87) yr ^b	62 (30-81) yr ^a
ASA (I/II/III)	18/13/5	23/25/10	14/15/6
Complicated cholecystitis	9	34 ^b	15
Preoperative ERCP	2	10	8 ^a
Spillage	21	39	18
Drain use	17	32	19
Operative time	55 (35-90) min	62.5 (25-120) min	72.5 (35-120) min ^a

^a*P* < 0.05, ^b*P* < 0.01 *vs* group I.

to compare the two groups of population. They included invariably patients who had surgery within the first 7 d for comparison. In our study, the patients were divided into three groups, including those who were treated surgically during the index admission even beyond one week from the onset of their illness.

Our findings are in accordance with previous studies, suggesting the safety of early LC for AC. The present study, however, does not support the findings of earlier reports, regarding the rising conversion rate, when LC for AC is performed after 72 h^[6,12,15]. Our data have shown that the timing of cholecystectomy does not influence the conversion rate, as recently shown by others^[8,10]. This is probably attributed to the very low conversion rate in the whole group of our patients, making any differences between the subgroups insignificant. Our total conversion rate of 4.6% for LC in AC is one of the lowest in the literature and only slightly higher than that in our team's experience with elective LC for the same period (~1%). Even one week after the onset of symptoms there was nothing to suggest increased risk with regards to the conversion rate and morbidity; this has never been challenged before. Another issue of concern in laparoscopic treatment of AC is the presumed increased risk of bile duct injury when the procedure is performed beyond the early edematous phase of the first 48-72 h. Our data do not support this traditional belief, as there was no major bile duct injury in any of the patients. It is possible that the majority of patients with AC who are deferred for interval LC because they are outside this “early window of chance” are faced with a “difficult” elective cholecystectomy after few weeks^[16]. Waiting for the gallbladder to “cool down” allows maturation of acute inflammation, neovascularization, fibrosis, and contraction, making the dissection more difficult, as it has been proposed by others^[9]. While inflammation in the early stages may not necessarily involve Calot's triangle structures, chronic inflammation may scar and distort it, making dissection in this critical area more difficult and prone to bile duct injuries.

In conclusion, our data show that LC for AC during the index admission is safe and associated with a low morbidity and a low conversion rate. These findings refer not only to those patients who undergo surgical treatment very

Table 3 Complications and their treatment

Group 1: ≤ 3 d (n = 36)	Group 2: 4-7 d (n = 58)	Group 3: ≥ 8 d (n = 35)
(1) Subhepatic collection Laparoscopic drainage	(2) Bile leaks ERCP and CBD stent Spontaneous closure at 48 h	(2) Bile leaks ERCP and CBD stent
(1) Bleeding Laparotomy d 1	(1) Subhepatic collections Percutaneous CT guided drainage Laparotomy after failed percutaneous	(1) Bleeding from drain site Drain removal
(1) Wound infection (converted) Wound opening	(1) Re-admission at postop day 15 with cholangitis ERCP & sphincterotomy (1) Chest infection Antibiotics, physiotherapy	(1) Severe pancreatitis ICU admission (1) Readmission at postop d 6 with DVT Heparin

Complications are presented with parentheses to indicate the number of patients suffered the complication; under the complication line the way of management for each case (without numbers) is presented.

early, but also to those treated after the window of the first 3 or 7 d from the onset of symptoms. Further prospective randomized trials focusing on this particular question are required to validate these results. However, it appears reasonable to state that in units with expertise in laparoscopic surgery, every effort should be made to operate on all patients with AC during the index admission as soon as diagnosis is made and co-morbidities are dealt with, regardless of the time delay from the onset of symptoms. This policy is safe, not associated with a higher conversion rate or morbidity and results in an overall shorter hospitalization by avoiding re-admissions.

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RAPID COMMUNICATION

Long-term outcomes of chronic hepatitis C patients with sustained virological response at 6 months after the end of treatment

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sponse; Long-term outcome

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Abstract

AIM: To assess the clinical, biochemical, and virological outcome during long-term follow-up of chronic hepatitis C patients with sustained virological response following effective antiviral therapy.

METHODS: This study was a retrospective cohort study including 171 sustained responders defined as HCV RNA PCR negative at 6 mo after the end of effective antiviral treatment (SVR-6). Clinical signs and symptoms, biochemical hepatic parameters, ultrasonography and HCV RNA PCR were followed.

RESULTS: Mean follow-up period was 35.38 ± 22.2 mo after the end of treatment. Twenty-seven (15.8%) responders had evidence of cirrhosis before treatment. Forty-eight (28.1%), 107 (62.6%) and 6 (3.5%) patients were genotype 1, 3, and 6 respectively, while 10 patients (5.8%) were unclassified. There were no virological and biochemical relapses during the period of follow-up. None of the patients showed evidence of hepatic decompensation. However, there were 3 patients (1.8%) developing hepatocellular carcinoma at 14, 18, 29 mo after treatment discontinuation, two of whom had evidence of cirrhosis prior to therapy.

CONCLUSION: The study shows that during a follow-up interval for about 3 years in 171 chronic hepatitis C patients with sustained viral response after effective antiviral treatment there were no evident signs of either biochemical or clinical relapse of liver disease in all but three patients who developed hepatocellular carcinoma.

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Key words: Chronic hepatitis C; Sustained virological re-

INTRODUCTION

Chronic hepatitis C viral (HCV) infection is well recognized as a major cause of cirrhosis and hepatocellular carcinoma. Spontaneous remission of the disease seems to be rare. After alpha-interferon was used as therapy for HCV infection in 1986^[1], Interferon associated with Ribavirin became the standard treatment for this disease. Sustained virological response, defined as HCV RNA PCR negative at 6 mo after the end of treatment (SVR-6), is considered to be a useful predictor for long term response^[2], since the probability of a late relapse among sustained responders was only 4.7%-8.7%^[2-5], and a sustained response is associated with decreased histological activity on liver biopsy^[6,12]. Moreover, the disappearance of detectable plasma HCV RNA has fostered the notion that sustained responders may be cured of the disease^[4].

From most of the studies, SVR-6 can be obtained in 40%-60% of individuals infected with genotype 1 and in a higher percentage (75%-85%) of subjects with genotypes 2 and 3^[5]. However, there are only few studies reporting long-term outcomes in patients who had SVR-6^[1-4,6-8], especially in Asian population. In this study, we assessed the long-term clinical, biochemical and virological outcome of Thai HCV patients with sustained virological response.

MATERIALS AND METHODS

Study design

Chronic hepatitis C patients with SVR-6 from Hepatology Unit, Faculty of Medicine, Mahidol University (Siriraj Hospital), Bangkok, Thailand from 1995 to 2005 were included in this retrospective cohort study.

Patients

The criteria required for the study included: evidence of

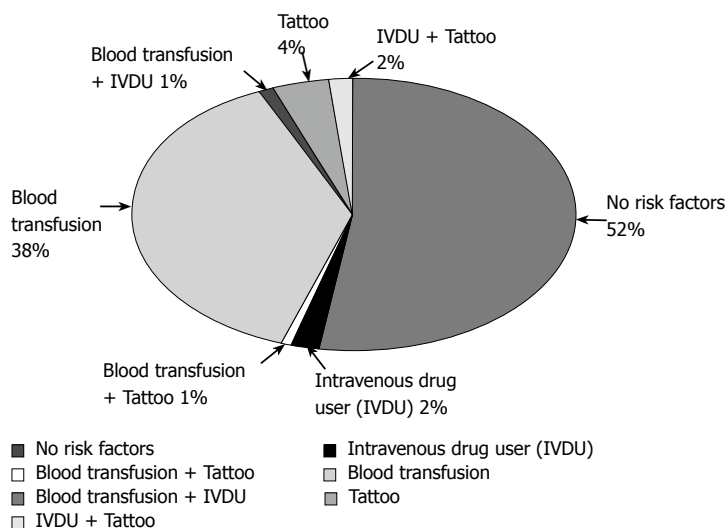


Figure 1 Routes of infection.

Table 1 Characteristics of 171 HCV infection patients with SVR-6 ($n = 171$)

Characteristics	
Age (yr)	
Mean \pm SD	48 \pm 10.5
Range	21-75
Sex	
Male	91 (53%)
Female	80 (47%)
Follow up (mo)	
Mean \pm SD	35.38 \pm 22.2
Range	8-134
Genotype	
1	48 (28.1%)
3	107 (62.6%)
6	6 (3.5%)
Unclassified	10 (5.8%)
Cirrhosis (before therapy)	27 (25.8%)
HCV viral load (copies/mL)	1 000-136 000 000
Median HCV viral load	903 000

chronic HCV infection as defined by HCV RNA PCR positive by Roche Amplicor HCV Assay[®] (with a detection limit of 100 copies/mL), age of 18 years or older, and sustained virological response after effective antiviral treatment. Patients co-infected by chronic hepatitis B virus and/or human immunodeficiency virus and patients where complete data could not be retrieved were excluded from the analysis.

Data were obtained from 171 chronic hepatitis C patients who responded to interferon therapy. Data were collected from case record forms. Patients treated with conventional interferon, PEG-interferon α -2a and PEG-interferon α -2b were 54.4%, 17% and 28.6% respectively. Twenty-one patients (12.3%) required therapy twice to achieve SVR-6. Duration of therapy was 24 wk for responders with genotype 3 infection, while non-responders and other HCV genotypes patients were treated for 48 wk.

Data recorded

The investigators recorded demographic data, details of treatments (type of interferon used and duration of therapy), virological data (genotypes and viral loads) and biochemical data (bilirubin, transaminases and albumin levels) obtained from certified laboratories. Pre-treatment liver biopsies were done in 105 of the 171 patients using the Histology Activity Index (HAI) score. Mean histological score and fibrosis score were 7.72 ± 4.08 (range 1 to 18) and 1.51 ± 1.3 (range 0 to 4) respectively.

Sustained virological response was defined as no detectable HCV RNA PCR at the end of treatment and after six months of follow up.

Patients were considered to have decompensation if they showed any of the following findings: ascites, bleeding varices, jaundice, or hepatic encephalopathy. Patients were classified as having cirrhosis on the basis of ultrasonography or liver biopsy. Hepatocellular carcinoma was diagnosed from ultrasonography or other imaging

studies.

Sustained virological responders were considered to have a late virological relapse if HCV RNA PCR became detectable on any occasion after six months of follow up, confirmed by HCV viral load study.

Follow-up data was recorded every six or twelve months for clinical, biochemical and virological outcomes.

Statistical analysis

Statistical analyses were performed using SPSS version 10 for Windows. The descriptive statistics and Paired *t*-Test were used to analyse. The Kaplan-Meier method was used to determine the rate of hepatocellular carcinoma occurrence.

RESULTS

Study population

Data were obtained from 171 patients treated for chronic HCV infection who had SVR-6. The ages of the patients were 48 ± 10.5 years. Male to female ratio was 1.12:1. Routes of infection are shown in Figure 1. Characteristics of sustained virological responders are shown in Table 1.

Of 171 sustained responders, 27 patients (15.8%) had compensated cirrhosis (as determined by liver biopsy or imaging study) before the start of treatment.

Clinical, biochemical and virological studies were followed up to 35.38 ± 22.2 mo (range 8 to 134 mo) after the end of treatment.

Clinical outcomes

At the end of follow-up, all patients were fully active and alive. No new cases of cirrhosis appeared. Of the 27 patients with cirrhosis, none developed decompensated liver disease. Hepatocellular carcinoma, as assessed by abdominal ultrasonography every 6 mo were found in three patients at 14, 18 and 29 mo of follow-up. The details of these patients are shown in Table 2. Their HCV RNA values were still undetectable at the time of the diagnosis

Table 2 Features of the three hepatocellular carcinoma patients

	Case1	Case 2	Case 3
Age (yr)	50	75	63
Sex	Male	Male	Male
Previous cirrhosis	Yes	Yes	No
Onset after the end of treatment (mo)	14	18	29
Alfa-fetoprotein (IU/mL)	433.7	2.09	21.59
Size at first detection	3 cm	1.5 cm	1 cm, 3 lesions

of hepatocellular carcinoma. Two of them had cirrhosis before treatment. From the statistical analysis of this data, it showed that, after a median follow-up period of 31 mo, the incidence of developing hepatocellular carcinoma in SVR-6 patients was no more than 1.8%.

Biochemical and virological outcomes

There was a significant reduction of aminotransferase levels after SVR-6. The pre-treatment and post-treatment values of AST were 84.3 ± 48.9 and 27.7 ± 12.9 , while ALT were 120.4 ± 81.2 and 25.2 ± 15.3 U/L (the normal values of AST: 0-37 and ALT 0-40 U/L).

No late virological relapse was found. Four (2.3%) of the 171 sustained responders had false positive HCV RNA PCR (confirmed by HCV RNA viral load less than 50 IU/mL).

DISCUSSION

We assessed the long term outcome of 171 patients with chronic hepatitis C who achieved sustained virological response by effective antiviral treatment. Our result demonstrated that SVR-6 was associated with a permanent absence of HCV viremia during the long-term follow-up in all cases. However, this finding showed better result than previous reports which had a late virological relapse between 4.7%-8.7%^[2,3]. The reasons may due to: (1) the smaller number of patients included compared to previous studied; (2) fewer HCV genotype 1 included compared; (3) more than half of our patients were infected by genotype 3 (this type is known to have a more favorable prognosis), and (4), the low sensitivity of HCV RNA PCR tests used in the studies before 2000 that may potentially account for few apparent early virological responses.

Disease progression such as development of cirrhosis seems to arrest after the stage of SVR-6, but a risk of developing hepatocellular carcinoma is still remaining. The incidence of hepatocellular carcinoma from our study at a mean follow-up period about 3 years is 1.8% which is slightly higher than the previous results reported from Japan (0.02%-0.5% per year)^[3,9,10], while the incidence of hepatocellular carcinoma in western countries seems to be rare after SVR and limited only to patients with cirrhosis^[4,11]. Therefore, regular ultrasonography should not be discarded for the management of cirrhotic patients, even in those showing persistently normal aminotransferase, alfa-fetoprotein and undetectable HCV RNA levels after interferon treatment.

In summary, this long-term study shows that in chronic

HCV infection, sustained responders to interferon attain remarkable improvement of clinical outcomes. This supports the hypothesis that persons with sustained responses to interferon-therapy show a low risk for further relapse of HCV infection, development of cirrhosis and hepatocellular carcinoma^[3,4,6,7,12]. However, the follow-up period of these patients was too short to allow a definite conclusion about the potential effect of the sustained response to interferon therapy for prevention of progressive liver disease.

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RAPID COMMUNICATION

Comparative study of two bowel preparation regimens for colonoscopy: Senna tablets vs sodium phosphate solution

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to sodium phosphate solution in bowel preparation for colonoscopy, but senna may be considered an alternative laxative.

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Key words: Senna tablet; Sodium phosphate solution; Colonoscopy

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Abstract

AIM: To compare the efficacy and acceptance of senna tablet and sodium phosphate solution for bowel preparation before colonoscopy.

METHODS: One hundred and thirty four patients, who needed elective colonoscopy, were randomly allocated to take 180 mg senna tablet or 95 mL sodium phosphate solution on the day before colonoscopy. The efficacies of both laxatives were compared using the mean difference of colon-cleanliness score of the rectum, sigmoid segments, descending colon, transverse colon and cecum. The scores were rated by two observers who were blinded to the laxatives administered. The higher score means that the colon is cleaner. The efficacy of both laxatives were equivalent if the 95% confidence interval of the mean difference of the score of colon lie within -1 to +1.

RESULTS: On intention-to-treat analysis, the mean cleanliness scores in the four segments of colon except the cecum were higher in the sodium phosphate group than those in senna group (7.9 ± 1.7 vs 8.3 ± 1.5 , 8.0 ± 1.8 vs 8.5 ± 1.4 , 7.9 ± 2.0 vs 8.5 ± 1.3 , 7.9 ± 2.0 vs 8.2 ± 1.4 and 7.2 ± 1.7 vs 6.9 ± 1.4 , respectively). The 95% confidence intervals (95% CI) of mean difference in each segment of colon were not found to lie within 1 point which indicated that their efficacies were not equivalent. The taste of senna was better than sodium phosphate solution. Also, senna had fewer side effects.

CONCLUSION: The efficacy of senna is not equivalent

INTRODUCTION

Adequately cleansed colon is essential for colonoscopy. Inadequate bowel preparation might lead to missed diagnosis, increasing the time of colonoscopy by 7.5%-10.3% and increasing cost 12%-22%^[1]. Ness *et al*^[2] reported that the incidence of inadequate bowel preparation was 21.7% and 5.4% had poor preparation leading to cancellation or abortion of procedure. Currently, the laxatives of choice for bowel preparation are sodium phosphate solution (NaP) and polyethylene glycol solution (PEG). Despite its efficacy^[3-5], phosphate solution has poor taste. It may cause electrolyte imbalance, severe nausea and vomiting. The advantage of PEG is its minimal effect on intravascular volume and serum electrolyte balance, but this large-volume laxative is difficult for many patients to tolerate. Although PEG and NaP are equally effective in colonic cleansing^[6], NaP is better tolerated. However, NaP may be contraindicated in certain patient populations. The selection of a colonoscopy preparation requires clinical judgment, cost and informed patient preference^[7,8].

Senna (*Cassia angustifolia* Vahl, *Leguminosae*, Indian senna, Tinnevely senna) is a laxative that stimulates the intestinal motility and affects epithelial transport of water and electrolytes. The main advantages of senna are low cost, safety and ease of ingestion. It had been combined with other laxatives for bowel preparation, and their efficacy ranged from 70% to 85%^[9-12]. There are few studies on the efficacy of high-dose senna tablet alone. The aim of

this study was to compare the efficacy and acceptance of senna tablet and sodium phosphate solution for bowel preparation before colonoscopy.

MATERIALS AND METHODS

The study was carried out as a randomized, controlled (equivalent), single-blind trial from June to November 2003. The study population consisted of adult patients who required elective colonoscopy. The exclusion criteria were: (1) known allergy to senna or sodium phosphate solution; (2) presence of severe metabolic, renal and cardiac conditions; (3) bed-ridden or psychotic patient; (4) pregnancy; (5) patient taking laxatives within one week prior to enrollment; and (6) patients who had previous colonic resection surgery. The study was approved by the Ethical Committee Board of the hospital.

The patients were allocated into two groups and they were advised to take full liquid diet two days before colonoscopy. The control group took sodium phosphate solution (Swift® 90 mL, Berlin Pharmaceutical Industry Co. Ltd., Thailand). The experimental group took senna tablet, 180 mg (24 tablets of 7.5 mg /tab, Senokot®, Reckitt Benckiser, Thailand). The patients took the laxatives in divided doses at 14.00 pm and 16.00 pm on the day before colonoscopy. Since the duration of action of NaP is within 6 h, so the laxatives should not interfere the patient's sleep-time.

Data collection

Shortly before colonoscopy, nurses interviewed each patient to assess compliance, acceptance and side effects of laxatives by using visual analog scale. The colonoscopist and his assistant independently rated the quality of bowel cleansing, using visual analog score (VAS) as followed: 0-2 = numerous solid feces, 3-5 = semi-solid feces, 6-7 large volume of liquid feces, 8-10 = small volume of clear liquid or no feces. In an equivalent trial^[13], it was important to pre-specified that (1) the mean score should lie above seven to assure that both laxatives were effective, and (2) the 95% confidence intervals of the mean difference lie between -1 and +1 VAS score in all segments of colon. The sample size calculation was based on testing equivalence with power 0.8 and 10% drop out^[14]. The variance of VAS score from our pilot study was 2.93.

Before data analysis, the 95% limit of agreement^[15] of cleansing score between two colonoscopists will be calculated to confirm the agreement on the assumption that the mean score difference between them should lie within two points. The score used for analysis were the average score from two colonoscopists. The VAS score for acceptance and side effects of the two laxatives were analyzed using Student's *t* test. The outcome variables of accepted and side effects of laxatives were also measured using VAS score. The patients were asked to grade the taste of the laxative as follows: 0-2 = hard to ingest, 3-5 = ingested with very bad feelings, 6-7 = easily ingested, and 8-10 = easily ingested with good feelings. The scores of the side effects (nausea and vomiting, abdominal pain, vertigo and sleeplessness) were rated as follows: 0-2 = no symptom, 3-5 = mild symptom, 6-7 = moderate symp-

Table 1 Demographic and baseline colonoscopic data of the patients (*n* = 67)

Characteristics of patients	Senna	NaP
Sex (M/F)	22 : 45	30 : 37
Age (yr, mean \pm SD)	54.3 \pm 12.7	51.6 \pm 12.6
Body weight (kg, mean \pm SD)	59.1 \pm 10.7	61.8 \pm 12.6
Constipation (Yes/No)	9:58	14:53
Laxative users (Yes/No)	6:61	12:55
Previous Obs-gyn surgery	6:61	7:60
Diabetes (Yes/No)	7:60	6:61
Colonoscopic diagnosis		
Normal study	44 (65.7%)	40 (62.5%)
Polyp	8 (12.1%)	4 (6.2%)
Diverticulosis	4 (6.0%)	8 (12.5%)
Carcinoma	4 (6.0%)	6 (9.3%)
Inflammatory bowel disease	6 (9.0%)	3 (4.6%)
Other	1 (1.5%)	3 (4.6%)
Time of colonoscopy (min, mean \pm SD)	19.3 \pm 14.2	18.2 \pm 10.1
Incomplete colonoscopy	4 (6.0%)	5 (7.8%)
Therapeutic:Diagnostic colonoscopy	16:51	14:50

Table 2 The cleansing score, acceptance score and side effects of laxatives (*n* = 67)

Segment	Senna (mean \pm SD)	NaP (mean \pm SD)	95% CI of differences
Rectum	7.9 \pm 1.7	8.3 \pm 1.5	-1.0 to 0.1
Sigmoid colon	8.0 \pm 1.8	8.5 \pm 1.4	-1.0 to 0.1
Descending colon	7.9 \pm 2.0	8.5 \pm 1.3	-1.2 to 0.0
Transverse colon	7.9 \pm 2.0	8.2 \pm 1.4	-0.9 to 0.3
Ascending colon and cecum	7.2 \pm 1.7	6.9 \pm 1.4	-0.2 to 0.8
Acceptance score			
Taste	8.6 \pm 1.9	5.1 \pm 2.8	<i>P</i> < 0.001
Side effects			
Nausea & vomiting	0.9 \pm 0.2	3.0 \pm 3.5	<i>P</i> < 0.001
Abdominal pain	1.3 \pm 2.3	1.4 \pm 2.4	<i>P</i> = 0.8
Vertigo	0.7 \pm 1.6	1.3 \pm 2.3	<i>P</i> = 0.08
Sleeplessness	1.2 \pm 2.7	1.4 \pm 2.5	<i>P</i> = 0.65
Adverse event	2:67	2:64	

tom, and 8-10 = severe symptom.

RESULTS

Patient's flow in this study is shown in Figure 1. One hundred and seventy patients were enrolled, but thirty patients did not meet eligibility criteria. One hundred and thirty-six patients were randomly allocated to take senna tablet or NaP solution. Two patients did not take laxatives due to error in packaging. Among the 134 patients who took a laxative, 3 patients did not attend colonoscopy and 10 patients did not have complete colonoscopy for various reasons.

Both groups of patients were comparable with regard to demographic data, diagnosis and other colonoscopic data (Table 1). However, the efficacy of senna tablet was not equivalent to NaP solution (Table 2). The mean

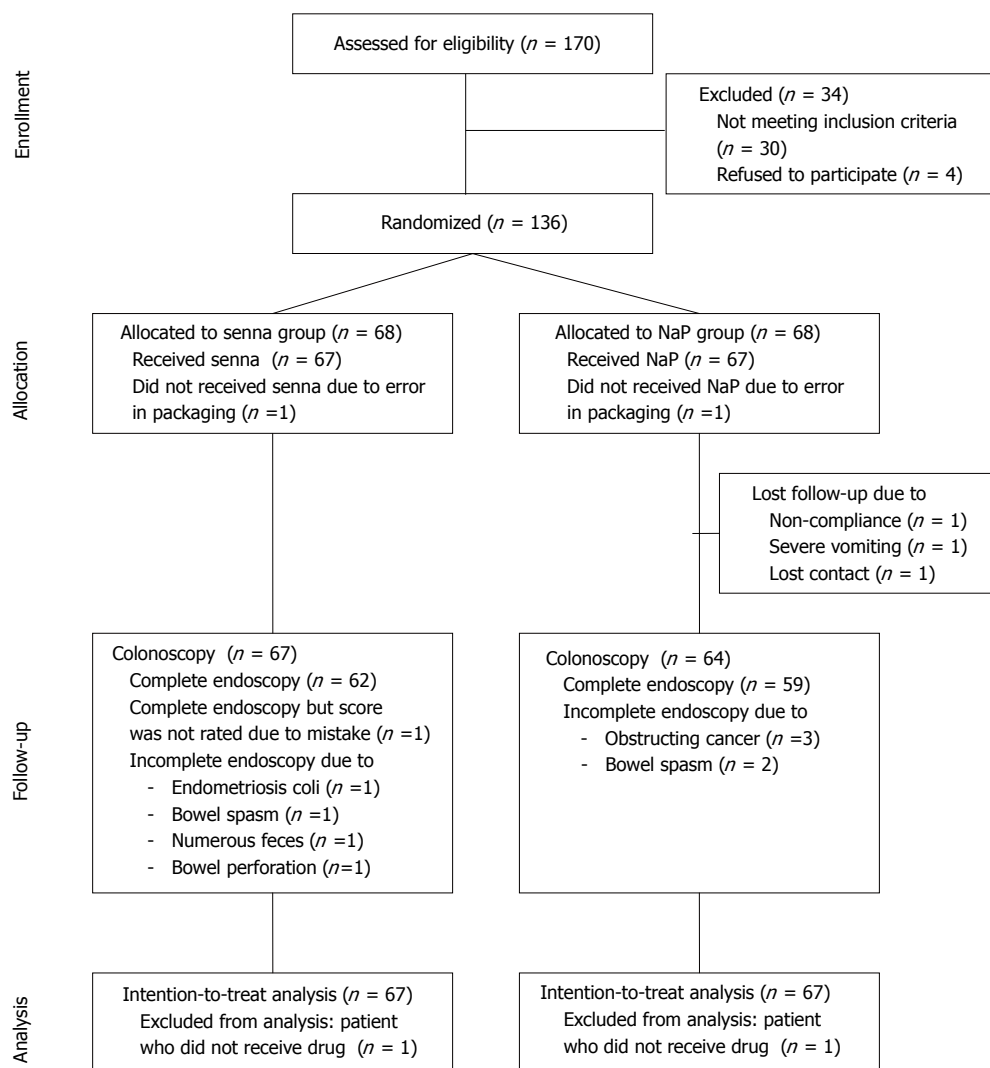


Figure 1 Flow diagram of patients progress through the phases of a randomized trial.

cleansing scores of NaP solution were higher than senna tablet in four segments of the colon except in the ascending colon and the cecum. The 95% CI of the mean difference exceeded 1 point in three segments of the colon. In the other two segments of the colon, they lied nearly over 1 point. By intention-to-treat analysis, we included all patients who had taken the laxatives whether they had complete or incomplete colonoscopy. For the missing data in both groups, we assigned the lowest score in each group (worst-case approach). For example, the lowest cleansing score in senna group was two, while that in NaP group was four.

The cleansing score, acceptance score and side effects of the two laxatives are shown in Table 2. The patients accepted senna tablets more than NaP solution and those patients who took senna tablets had less nausea and vomiting. There were four adverse events in this study. In the senna group, 1 patient had post-polypectomy bleeding which ceased spontaneously, and 1 patient had sigmoid perforation during colonoscopy due to fixation of the sigmoid colon; this patient had received long-term steroid treatment of myasthenia gravis and also had previous left hip surgery. In the NaP group, 2 patients had broncho-

spasm after colonoscopy and both recovered after 24 h.

DISCUSSION

NaP solution and PEG had widely been used for bowel preparation because of their similar efficacy, Hwang *et al*^[16] claimed that NaP group had higher completion rate than PEG group (84.2% *vs* 27.5%, $P < 0.001$) and NaP appeared to be more cost-effective^[16]. In contrast, senna was not popular for bowel preparation. Fear of adverse effects might responsible for its underuse. Serious adverse effects of senna, such as asthma, hepatitis, hypertrophic osteoarthropathy, cachexia, hypo-gammaglobulinemia, finger clubbing and tetany, had been reported^[17-21]. However, these adverse effects were uncommon and resulted from long-term and large amount used. There are no epidemiologic data to support neoplastic potential of senna compound^[22]. The inconsistent efficacy of senna might be another reason for its underuse. Two studies by Chilton *et al*^[11] and Valverde *et al*^[23] showed that senna (X-prep) solution alone or senna in combination with other laxatives were better than PEG or NaP solution. On contrary, two other studies by Dahshan *et al*^[24] and Arezzo *et al*^[12] showed that

those standard laxatives were better than senna. Moreover, Hangartner *et al*^[9] and Borkje *et al*^[10] concluded that senna has no clinical difference compared with those laxatives. Radaelli *et al*^[25,26] had claimed that high-dose senna had 97.3% efficacy in bowel cleansing, and that 288 mg of senna was better than 4 L of PEG-ES (90.6% *vs* 79.7% efficacy, *P* = 0.003). In contrast, our study showed that 180 mg of senna tablets did not have equivalent efficacy as NaP solution. The inconsistencies of these results were hardly explained. Bowel cleansing may be affected by other factors, such as gender, age, obesity, race, constipation, previous abdominal surgery and associated complicated diverticular disease^[27]. In addition, the mean score of senna group was also above seven points and we imply that senna has some effect in bowel cleansing and it may be alternative laxative for bowel preparation.

In addition, we noticed that the mean cleansing score of cecum in the senna group was higher than that in the NaP group. This phenomenon might be related to timing of laxative intake. Church *et al*^[28] suggested that the patients who took laxatives 5 h before colonoscopy had better result than patients who took laxative 1 d before colonoscopy. The VAS scores of taste, nausea and vomiting in the senna group were significantly better compared with the NaP group (Table 2). However, in term of pain symptom, senna was not found to be better than NaP. These findings confirmed our rational background knowledge that senna had more palatability and less nausea and vomiting than NaP solution. The adverse events occurred in 4 patients were not related to laxatives but were related to colonoscopy or anesthetic procedure.

In conclusion, senna does not have the same efficacy as oral NaP solution. However, senna has better compliance and fewer side effects than NaP. Senna may be prescribed as an alternative laxative for bowel preparation in patients who have contraindications to NaP solution.

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RAPID COMMUNICATION

Acute effect of smoking on gallbladder emptying and refilling in chronic smokers and nonsmokers: A sonographic study

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Abstract

AIM: To ultrasonographically evaluate the acute effects of smoking on gallbladder contraction and refilling in chronic smokers and nonsmokers.

METHODS: Fifteen chronic smokers (21-30 years old) and fifteen nonsmokers (21-35 years old) participated in this study. Chronic smokers were selected among the volunteers who had been smoking for at least 5 years and 10 cigarettes per day (mean 17.5/d). Examinations were performed in two separate days. In the first day, basal gallbladder (GB) volumes of volunteers were measured after 8-h fasting. After the examinations, participants had a meal containing at least 30-40 gram fat. Gallbladder volume was assessed at 5, 15, 30, 60, 120 and 180 min after the meal. In the second day, participants smoked 2 cigarettes after 8-h fasting. Then, they had the same meal, and gallbladder measurements were repeated at the same time points. Same procedures were applied to both groups.

RESULTS: The mean starving GB volumes were 23.3 ± 3.3 mL in the first day, 21.9 ± 3.0 mL in the second day in nonsmoker group and 18.3 ± 3.0 mL in the first day, 19.5 ± 2.8 mL in second day in smoker group. There was no significant difference between starving GB volumes. We did not find any significant difference between the GB volumes measured at 5, 15, 30, 60, 120 and 180 min in the first and second days in nonsmoker group. In smokers, post cigarette GB volume was found significantly higher at 5, 15 and 30 min which corresponded to GB contraction phase ($P < 0.05$). Control GB volume measurements were not significantly different between the two groups. Post-smoking GB volumes were also not significantly different between the two groups.

CONCLUSION: Smoking prolongs the maximal GB emptying time both in smokers and in nonsmokers though it is not significant. It delays GB contraction in

chronic smokers and causes a significant decrease in GB emptying volume. Smoking causes no significant delay in GB refilling in both smokers and nonsmokers. These effects of smoking observed in acute phase result in bile stasis in GB. Bile stasis is the underlying cause of most GB disorders in chronic process.

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Key words: Gallbladder; Emptying; Smoking; Ultrasonography

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INTRODUCTION

Smoking is still widespread in many societies despite the recognized relationship of it with many diseases. It is one of the risk factors for lung, stomach, larynx, esophagus, and some other cancers. Its association with lung cancer has been well described. Besides, smoking carries an important risk of developing cardiovascular diseases^[1]. Chronic pulmonary diseases, gastric ulcers, and many other diseases are increased among smokers. Some reports showed that smoking increases the risk of gallbladder cancer^[2]. Many prospective studies have found an association between smoking and clinical gallbladder disease^[3-6]. On the other hand, no relation has been found between smoking and gallstone formation in some other reports^[7,8]. A population-based study reported that sonographically detected gallbladder diseases are associated with smoking and are moderately increased in smokers^[9]. Some epidemiologic researches focused on the relation of smoking and gallbladder diseases, but they have not found any positive relation^[10,11]. There are a limited number of studies in the literature evaluating the effect of smoking on gallbladder motility by ultrasonography. Janderko *et al*^[12] evaluated gallbladder contraction and refilling in chronic smokers by ultrasonography and found that refilling is delayed. In our study, we evaluated the gallbladder volume changes in contraction and refilling periods in both smokers and nonsmokers by

ultrasonography. We compared the obtained data of both groups. To our knowledge, there is no other study in the literature evaluating the effects of smoking on gallbladder in the acute period in smokers and nonsmokers by ultrasonography.

MATERIALS AND METHODS

Subjects

Fifteen smoker (10 women and 5 men) and 15 nonsmoker volunteers (9 men and 6 women) were included in the study. The mean age of smokers was 24.2 years (21-30 years), and the mean age of nonsmokers was 28.1 years (21-34 years). Volunteers having smoked for at least 5 years were included in group of smokers. The mean smoking rate in this group was 17.5 cigarettes per day (10-25). None of the volunteers had gastrointestinal disorder, gallbladder disease, any disease like diabetes mellitus that could affect gallbladder, or any previous surgery of gastrointestinal tract. Prior to 48-h and during the study, no medicine or smoking was allowed. A preliminary gallbladder sonography was performed and only the volunteers without any gallbladder abnormality were included in the study. All participants gave their informed consent.

Study design

Gallbladder volumes of all volunteers were measured twice in two separate days. At least 8 h after the last meal, participants were taken into the dimly lighted sonography room at a temperature of 22-24°C. A 3.5 6 MHz convex broadband (tissue harmonic) abdominal transducer (Toshiba, Nemio, Tokyo-Japan) was established for the measurements. All measurements were performed in supine, left lateral, and lateral decubitus positions in which gallbladder cross-sectional and longitudinal diameters were best visualized. The best imaging position of gallbladder was recorded for each participant and used in following measurements. The most appropriate transducer position was noted for each volunteer and marked on the skin. Cross-sectional diameters were calculated from the widest diameters measured during sonography. The longest axis of the gallbladder was established as the longitudinal diameter. The distance between internal margins of each opposite wall was used for the measurement of diameters. The subjects with significant folding of gallbladder or the subjects whose longitudinal diameter could not be viewed in a single frame were excluded from the study.

Longitudinal and cross-sectional diameter measurements were repeated three times and the mean of these three measurements was established. Gallbladder volume was calculated with the formula $V = (\pi/6) \times L \times W \times H$, where V = GB volume, L = GB length, W = GB width, H = GB height, as previously described^[13].

Study methods

Gallbladder volumes of all volunteers were measured twice in two separate days. In the first day (control examination) after 8-h (at least) overnight fasting, basal GB volumes were measured. Then, 30-40 g fat-containing meal was given to the volunteers and participants were told to eat the

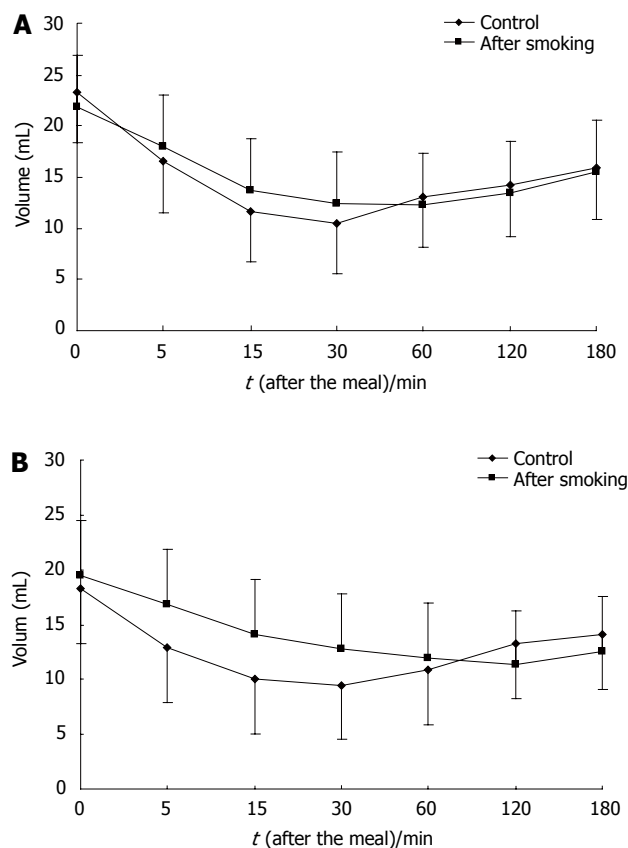


Figure 1 Effect of smoking on gallbladder emptying and refilling in nonsmokers (A) and smokers (B).

meal up in 2 min. After that, GB volumes were measured at 5, 15, 30, 60, 120 and 180 min. Contraction phase as the measurements till 60 min, and refilling phase was presumed as the measurements at 120 and 180 min. To avoid the start of cephalic phase of digestion, volunteers and meals were kept in separate rooms until the examinations. In the second day (study examination), after an overnight fasting, two cigarettes were given to the participants who were encouraged to consume the cigarettes in 5-10 min with deep inhalations. Approximately 3 min after the smoking, a meal containing 30-40 g fat, was given to the volunteers. Then, GB volumes were measured at 5, 15, 30, 60, 120 and 180 min. The same procedures were repeated in smoker and nonsmoker groups.

GB volume changes versus time were presented graphically for both two groups using the pre- and post-smoking values (Figure 1A and B). Acquired data are shown on Tables 1 and 2 by comparing them between the two groups and within each group.

Statistical analysis

Values are presented as mean \pm SD. The paired and two-tailed Student's *t* tests were used. $P < 0.05$ was considered statistical significant.

RESULTS

There was no difference in body mass index and age between smokers and nonsmokers. The mean fasting GB

Table 1 Postprandial gallbladder volumes after smoking in smokers and non-smokers (mean \pm SD, mL)

t/min	Non smoker			Smoker		
	Control	After smoking	<i>P</i>	Control	After smoking	<i>P</i>
0	23.3 \pm 3.3	21.9 \pm 3.0	0.175	18.3 \pm 3.0	19.5 \pm 2.8	0.371
5	16.5 \pm 2.9	18.0 \pm 2.8	0.174	12.9 \pm 2.1	16.9 \pm 2.5	0.022
15	11.7 \pm 2.5	13.7 \pm 2.6	0.080	10.0 \pm 1.5	14.1 \pm 1.6	0.011
30	10.5 \pm 1.9	12.4 \pm 2.1	0.432	9.5 \pm 1.6	12.8 \pm 1.7	0.042
60	13.1 \pm 2.4	12.3 \pm 2.0	0.756	10.9 \pm 1.5	12.0 \pm 1.6	0.359
120	14.2 \pm 2.5	13.5 \pm 1.8	0.859	13.3 \pm 2.9	11.3 \pm 1.5	0.398
180	15.9 \pm 2.5	15.5 \pm 1.9	0.508	14.1 \pm 2.9	12.6 \pm 1.7	0.449

volume in nonsmoker group was 23.3 ± 3.3 mL in the first day and 21.9 ± 3.0 mL in the second day, and was 18.3 ± 3.0 mL in the first day and 19.5 ± 2.8 mL in the second day in smoker group. Although the fasting GB volumes did not differ significantly, these values were lower in smoker group. In the first day, minimum GB volumes (in other words, maximal GB emptying) were measured at 30 min in both groups.

However, the post-smoking measurements in the second day were performed at 60 postprandial minute in nonsmoker group, and at 120 min in smoker group (Figures 1A and 1B). Smoking delayed the maximal GB emptying in both smokers and nonsmokers. Besides, in both groups, GB refilling was faster in the first day and slowed down in the second day (Figure 1A and B). In nonsmoker group, GB volumes measured at 5, 15, 30, 60, 120 and 180 min in the first and second days were not significantly different (Table 1). GB volume was significantly higher in smoker group at GB contraction phase (5, 15 and 30 min) after the two cigarettes were smoked ($P < 0.05$, Table 1). There was no significant difference between two groups both in basal GB volume measurements and in post-smoking measurements between the two groups (Table 2).

DISCUSSION

In this study, we showed the disrupted GB contractility in smokers and delayed maximal GB emptying both in smokers and nonsmokers just after smoking. Although our study group was small in size, basal GB volume had a tendency to be lower in smokers than in nonsmokers. However, this difference was not statistically significant. Many epidemiologic studies have reported a mild or moderate association between smoking and gallstone or postcholecystectomy state^[4,6,10,11]. On the other hand, no association has been detected between smoking and gall stone formation^[7,8,14]. It is not clear which biologic mechanism is mediated in the predisposition of smoking to gallstone formation. Some authors suggested that smoking lowers the plasma high-density lipoprotein cholesterol level which increases risk of gall stone formation by decreasing hepatic excretion of the bile acids^[15]. Estrogen is blamed for gall stone formation because of high incidence of gallstone in women. In respect to this, some studies have shown high plasma estrogen level in smokers^[16], while some others have not found any significant difference^[17]. Cholecystokinin, a proximal gut hormone, is a well

Table 2 Postprandial gallbladder volumes after smoking in smokers and non-smokers (mean \pm SD, mL)

t/min	Control			After smoking		
	Non smoker	Smoker	<i>P</i>	Non smoker	Smoker	<i>P</i>
0	23.3 \pm 3.3	18.3 \pm 3.0	0.360	21.9 \pm 3.0	19.5 \pm 2.8	0.604
5	16.5 \pm 2.9	12.9 \pm 2.1	0.392	18.0 \pm 2.8	16.9 \pm 2.5	0.783
15	11.7 \pm 2.5	10.0 \pm 1.5	0.623	13.7 \pm 2.6	14.1 \pm 1.6	0.883
30	10.5 \pm 1.9	9.5 \pm 1.6	0.732	12.4 \pm 2.1	12.8 \pm 1.7	0.883
60	13.1 \pm 2.4	10.9 \pm 1.5	0.513	12.3 \pm 2.0	12.0 \pm 1.6	0.909
120	14.2 \pm 2.5	13.3 \pm 2.9	0.839	13.5 \pm 1.8	11.3 \pm 1.5	0.318
180	15.9 \pm 2.5	14.1 \pm 2.9	0.701	15.5 \pm 1.9	12.6 \pm 1.7	0.295

known mediator of GB contraction^[18]. It is the main determining factor for the postprandial GB discharge. Cholecystokinin starts GB contraction by affecting the pre-ganglionic cholinergic nerves. Cholecystokinin is released from mucosa cells by the arrival of stomach content that is rich in fat and protein to the small intestine^[19]. Smoking exerts an inhibitory effect on intestinal and gastric motility^[20-22]. As a result of decreased gut motility and delayed gastric emptying, cholecystokinin release is also decreased or delayed. Therefore, decreased GB contractility can be expected. However, in our study, smoking caused a nonsignificant and minimal decrease of GB contractility in nonsmoker volunteers. In addition, GB volume was slightly increased in post-smoking contraction phase in this group (Figure 1A). Previous studies have also reported similar results^[23]. On the other hand, we showed a significant difference of GB volume in post-smoking early contraction phase (postprandial 5, 15 and 30 min) of smokers (Table 2). It was also significantly higher in post-smoking 5, 15 and 30 min (Figure 1B). To the best of our knowledge, no data are available on whether this delay of GB contraction in smokers results from a disruption in the smooth muscle contraction mechanism of GB wall or from the delay of the blood cholecystokinin increase due to delayed gastric emptying. One of the limitations of this study is that the blood levels of cholecystokinin were not measured. According to Jonderko *et al*^[12], smoking delays GB refilling at acute phase in chronic smokers. Our results also showed that GB refilling was delayed after smoking both in smokers and in nonsmokers although not significant. Smoking suppresses pancreatic polypeptide release which plays an important role in GB refilling^[24,25].

In humans, disruption of GB emptying is associated with gallstone formation^[26]. Bile stasis and disrupted GB motility are important factors for gallstone formation^[27,28]. Delayed gallbladder emptying and reduced muscle contractility occur in chronic calculus, or in gallstone, cholecystitis^[29,30]. It was reported that acalculous cholecystitis is formed after inhalation of intense cigarette smoke in dogs^[31]. Impairment of gallbladder contractility may contribute to the clinicopathology of acalculous cholecystitis^[32].

In our study, smoking caused a nonsignificant prolongation of the maximal GB emptying time both in smokers and in nonsmokers at acute phase, delayed GB contraction in smokers, and decreased the GB emptying volume. Besides, smoking did not delay GB refilling

significantly in both groups. Because of all these acute effects, smoking comprises a risk for GB diseases. In the chronic process, smoking may have these effects by affecting GB smooth muscle contraction or by decreasing cholecystokinin release *via* the inhibition of intestinal motility, or by both. *In vitro* studies are needed to evaluate the effects of smoking on gallbladder smooth muscles and laboratory studies are required to show its effects on cholecystokinin release.

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RAPID COMMUNICATION

Furazolidone-based triple therapy for *H pylori* gastritis in children

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rate seems to be higher in patients with duodenal ulcer.

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Abstract

AIM: To evaluate the furazolidone-based triple therapy in children with symptomatic *H pylori* gastritis.

METHODS: A prospective and consecutive open trial was carried out. The study included 38 patients with upper digestive symptoms sufficiently severe to warrant endoscopic investigation. *H pylori* status was defined based both on histology and on positive ¹³C-urea breath test. Drug regimen was a seven-day course of omeprazole, clarithromycin and furazolidone (100 mg, 200 mg if over 30 kg) twice daily. Eradication of *H pylori* was assessed two months after treatment by histology and ¹³C-urea breath test. Further clinical evaluation was performed 7 d, 2 and 6 mo after the treatment.

RESULTS: Thirty-eight patients (24 females, 14 males) were included. Their age ranged from 4 to 17.8 (mean 10.9 ± 3.7) years. On intent-to-treat analysis (*n* = 38), the eradication rate of *H pylori* was 73.7% (95% CI, 65.2%-82%) whereas in per-protocol analysis (*n* = 33) it was 84.8% (95% CI, 78.5%-91%). All the patients with duodenal ulcer (*n* = 7) were successfully treated (100% vs 56.2% with antral nodularity). Side effects were reported in 26 patients (68.4%), mainly vomiting (14/26) and abdominal pain (*n* = 13). Successfully treated dyspeptic patients showed improvement in 78.9% of *H pylori*-negative patients after six months and in 50% of *H pylori*-positive patients after six months of treatment.

CONCLUSION: Triple therapy with furazolidone achieves moderate efficacy in *H pylori* treatment. The eradication

INTRODUCTION

Available treatment regimens for gastritis due to *H pylori* show a lower success rate in children than in adult patients in the same geographic region. Several factors influence *H pylori* eradication rate, such as compliance with treatment, mutations generating resistances, sanctuaries (sites where there is no contact between the bacterium and antimicrobial drugs), deficiency in immunity of the host, low gastric pH, large infecting load, dormant forms and reinfection^[1]. *H pylori* treatment in children has specific difficulties and the success rate of the current options is worse than that in adults. It is speculated that in children there is less compliance with treatment and the prevalence of resistant strains is higher due to the greater exposure to antibiotics because of common childhood diseases. In addition, patients without peptic ulcer seem to present a lower success rate^[2].

The ideal treatment regimen for *H pylori* eradication should present a higher than 80% cure index on intention-to-treat analysis. Antimicrobial resistance is a major concern and in the past decade there was the emergence of clarithromycin-resistant strains, reaching 34.7% of isolates in some, mainly developed countries^[3-5]. Resistance occurs due to punctual mutations in the 23S rRNA^[5]. Nevertheless, in developing countries, resistance to metronidazole is highly prevalent, possibly due to overuse of this antimicrobial drug in gynecology and parasite treatment; thus this drug is not a viable alternative^[6]. Although amoxicillin resistance is regarded a rare phenomenon, recently there are an increasing number of

reports on resistant strains^[7].

Furazolidone emerges as an alternative for therapeutic regimens in developing countries due to its low cost and prevalence of resistant strains. This antimicrobial is a monoamine oxidase inhibitor usually utilized in the treatment of giardiasis. There are studies demonstrating its efficacy and safety in several developing countries^[8-12]. The drug has been used in *H pylori* treatment regimens since 1990, initially tested in China with a reasonable success rate and constitutes an alternative in situations where there is high resistance prevalence to nitroimidazoles. In our country a triple regimen with furazolidone, clarithromycin and omeprazole can attain a 90% cure in adult patients on intention-to-treat analysis^[13] while with furazolidone, levofloxacin and rabeprazole can reach 83% eradication as a third-line regimen^[14]. Consensus statements providing guidelines for the management of *H pylori* infection in children have made recommendations for therapy based on data derived from adult trials but have not provided suggestions on therapeutic options^[15,16]. The present study was to evaluate the triple regimen with omeprazole, clarithromycin and furazolidone for 7 d in children with *H pylori* gastritis, being the first study in children.

MATERIALS AND METHODS

Subjects

To warrant *H pylori* eradication, patients meeting the following criteria were included: (1) duodenal ulcer or erosive duodenitis ($n = 7$); (2) ulcer-like functional dyspepsia, according to the Rome II criteria, sufficiently severe to justify upper gastrointestinal endoscopy and without major mucosal abnormalities ($n = 29$)^[17]; (3) upper gastrointestinal bleeding ($n = 1$); (4) iron-deficiency anemia refractory to standard treatment ($n = 1$). Patients with former unsuccessful treatment for *H pylori* or with other organic diseases that could explain the symptoms were excluded. The study was approved by the Ethics Committee of the “Universidade Federal de São Paulo/Escola Paulista de Medicina”. On inclusion of the patients in the study, the responsible person(s) received written information about the patients and signed a free and informed consent.

Diagnosis of infection

Endoscopic examination was performed by our team, under deep sedation or general anesthesia supervised by an anesthetist. Four biopsy specimens were collected from the gastric antrum at approximately 2 cm from the pylorus, two for rapid urease test and two for histological analysis. The latter two were fixed in 100 mL/L formol, placed on filter paper and stained with hematoxylin-eosin and modified Giemsa. The findings were described according to modified Sydney criteria^[18]. The histological diagnosis of the infection was established by an experienced pathologist, based on the typical appearance of the bacterium along the mucus layer covering the gastric mucous membrane. Rapid urease test was performed with a non-commercial solution (100 mg/mL aqueous urea solution with 10 mg/mL phenol red) as previously described^[19]. The patient

Table 1 Endoscopic findings

Endoscopic findings	n	%
Nodular gastritis	16	42.1
Normal	13	34.2
Duodenal ulcer or erosive bulbitis	4	10.5
Erosive duodenitis and nodular gastritis	1	2.6
Erosive gastritis	1	2.6
Nodular gastritis and esophagitis	1	2.6
Duodenal ulcer and esophagitis	1	2.6
Erosive duodenitis, esophagitis and nodular gastritis	1	2.6

was considered infected when both tests were positive and non-infected when both were negative.

H pylori treatment

The triple regimen was administered twice daily for seven days: 100 mg furazolidone or 200 mg furazolidone (> 30 kg), 250 mg clarithromycin or 500 mg clarithromycin (> 30 kg), 10 mg omeprazole or 20 mg omeprazole (> 30 kg). Antibiotics were prescribed after meals whereas omeprazole was administered before the first meal. Patients and their responsible persons were advised to maintain the treatment even with minor adverse effects. On the last day of the treatment, a complete physical examination was performed to evaluate the clinical conditions of patients. During this examination the patients were asked about adverse effects, and compliance was controlled by return of empty medication blisters. Compliance with treatment was defined by over 75% intake of the prescribed doses.

H pylori eradication

A renewed clinical and endoscopic evaluation was performed two months after the treatment, with collection of antrum and corpus biopsies for histology and rapid urease test. Patients whose responsible persons did not give consent to another endoscopy were evaluated using the ¹³C-urea breath test. This test was performed as previously described^[20]. The cutoff value of breath test was delta over baseline 4‰. The patients were submitted to a new clinical evaluation two and six months after treatment and asked about the progress of symptoms and the frequency and intensity of epigastric pain in those with dyspepsia.

Statistical analysis

Continuous variables were expressed by calculation of the mean and standard deviation. The eradication rates were expressed by calculation of the proportion with an 85% confidence interval (95% CI). Treatment groups were compared using Pearson's chi-square test with Fisher's exact test when necessary. Factors associated with treatment success were evaluated by estimation of the odds ratio with 95% confidence interval. $P < 0.05$ was considered statistically significant.

RESULTS

Thirty-eight patients were included (24 females) with their

Table 2 Adverse effects reported by 26 of the 38 patients

Adverse effect	n	%
Vomiting	14	36.8
Abdominal pain	13	34.2
Metallic taste	6	15.8
Diarrhea	5	13.2
Nausea	5	13.2
Dizziness	2	5.3
Headache	2	5.3
Asthenia	1	2.6
Skin rash	1	2.6

age ranging from 4 to 17.8 (mean 10.9 ± 3.7) years. Results of the endoscopic examinations are shown in Table 1. The histological analysis showed active chronic gastritis in all patients. Intensity of the neutrophil infiltrate was low in 9 patients (23.7%), moderate in 19 (50%) and intense in 10 (26.3%). Intensity of bacterial density on histology was low in 11 patients (28.9%), moderate in 19 (50%) and intense in 8 (21.1%).

Slight side effects were reported in 26 patients (68.4%), disappearing with the interruption of the treatment (Table 2). Compliance with the protocol occurred in 33/38 patients (86.6%), intake of medications was not correct in 4 patients and control of treatment was very late in one. The eradication rate of infection was 84.8% in 28/33 patients treated according to the protocol (95% CI: 78.5%-91%), and 73.7% by intent-to-treat analysis in 28/38 patients (95% CI: 65.4%-82%). Influence of demographic, clinical and histologic data on the success of treatment is shown in Table 3. The infection was eradicated in all the 7 patients with erosive duodenitis or duodenal ulcer, while only 9/16 (56.2%) patients with nodular antrum gastritis as a single alteration were successfully treated ($P = 0.08$).

Evaluation of *H. pylori* eradication was performed through histology and ^{13}C -urea breath test in 26 patients who were successfully treated and breath test in 2 patients who were successfully treated. *H. pylori* eradication evaluation was not performed in 4 patients whose medication intake was less than 25% of that prescribed. Results of endoscopy were normal in 21 (65.6%), nodular antrum gastritis in 9 (28.1%) and erosive gastritis in 1 (6.5%). Cure of infection was achieved in two patients with erosive gastritis. After the treatment, among the 26 cured patients, 9 (34.6%) had normal histology, 7 (26.9%) inactive chronic gastritis, 9 (34.6%) low neutrophil infiltrate and 1 (3.8%) moderate neutrophil infiltrate. Among the 6 patients remaining infected, a second endoscopy revealed low neutrophil infiltrate in 4 (67%) and moderate neutrophil infiltrate in 2 (33%), decreased neutrophil infiltrate in 3 (50%). Gastritis activity did not worsen in any of the patients.

Clinical progress

Success treatment of *H. pylori* infection in patients with functional dyspepsia is shown in Figure 1. After two months of treatment, 63% of the eradicated dyspeptic patients and 60% of the non-eradicated patients reported

Table 3 Influence of clinical and histological variables on therapeutic success

	Eradication rate (%)	Odds ratio ¹	95% CI	P
Demographic data				
Female gender	66.7	0.33	0.03-2.18	0.27
Age ≤ 10 yr	66.7	0.56	0.1-3.11	0.47
Indication for treatment				
Ulcer-like functional dyspepsia	65.5	0.00	0.0-1.23	0.08
Initial endoscopy				
Normal examination	76.9	1.30	0.23-9.44	1
Nodular gastritis ²	56.2	0.24	0.03-1.39	0.08
Duodenal ulcer or erosive bulbitis	100	undefined	undefined	0.16
Histology				
Intense activity	70.0	0.70	0.13-5.97	1
Moderate activity	78.9	1.73	0.32-10.17	0.46
Light activity	66.7	0.67	0.10-5.28	0.68
Intense density	75.0	1.09	0.15-13.19	1
Moderate density	73.7	1.00	0.18-5.45	1
Light density	72.7	0.93	0.16-7.01	1
Adverse effects				
Yes	76.9	1.67	0.27-9.37	0.69

¹ for therapeutic success; ² without duodenal ulcer or erosive duodenitis.

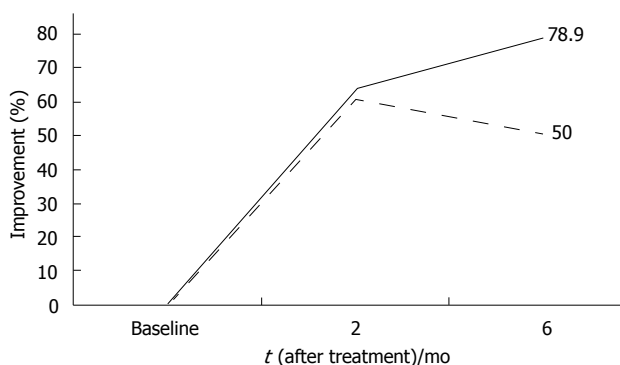


Figure 1 Patients with non-ulcer dyspepsia reporting symptom improvement after treatment according to *H. pylori* infection eradication in 2- and 6-mo follow-up. Continuous line: successfully eradicated patients; Dashed line: not eradicated patients. P (2 mo) = 1, P (6 mo) = 0.2.

improvement of symptoms ($P = 1$), while at 6-mo follow-up 78.9% successfully treated patients and 50% of the non-eradicated patients reported improvement of symptoms ($P = 0.2$). The hematological parameters of the patients with refractory iron deficiency anemia returned to normal after treatment and all patients with duodenal ulcer or erosive duodenitis were asymptomatic.

DISCUSSION

The attained success rate (73.3% by intent-to-treat analysis) was higher than that observed by a former study with amoxicillin, clarithromycin and omeprazole for 7 d (50%, 95% CI: 19%-81%) in our service, but similar to that for 10 d (73%, 95% CI: 51%-95%)^[21]. There is no other study

comparing triple therapy for 7 d with a longer treatment period. Other studies with clarithromycin, amoxicillin and proton pump inhibitor reported that the eradication rate of *H pylori* is 54%-77.8% in children^[6,22,23]. At present, the first-line regimen recommended by Brazilian consensus for the treatment of adults is the triple treatment with clarithromycin, amoxicillin (or furazolidone) and proton pump inhibitor for 7-14 d^[24]. A seven-day treatment period was chosen in our study because it is as effective as a ten-day period. The efficiency of a longer treatment period (14 d) is only 9% higher with a significant cost increase^[25]. In Brazil there are 16% clarithromycin-resistant and 55% metronidazole-resistant strains, thus requiring alternative regimens for classical compositions^[25,26]. Recently a sequential therapy has been described, consisting of two treatment regimens for five consecutive days. In these studies, amoxicillin and proton pump inhibitor (PPI) are used for five days followed by PPI, clarithromycin and tinidazole for another five days^[27]. In children the regimen is more efficient than the traditional treatment with amoxicillin, metronidazole and PPI for 10 d (97.3%, 95% CI: 86.2%-99.5% *vs* 75.5%, 95% CI: 59.8%-86.7%), and has no more side effects (global rate 12%)^[28]. A sequential regimen exposes the patients to three different drug classes as a first line treatment, which may make the choice of a second-line treatment difficult in eventual therapeutic failures.

There are no clinical or laboratory factors associated with a better result of the treatment (Table 3). The eradication rate of *H pylori* observed in our study in patients with duodenal ulcer is similar to that observed by Dani and coworkers^[13] in adults with ulcer disease, but the difference in the eradication rate of *H pylori* in patients with functional non-ulcer dyspepsia did not reach statistical significance, perhaps due to the small number of patients with ulcer included in our study ($P = 0.08$). A recent study has shown a lower eradication rate of *H pylori* in patients with non-ulcer dyspepsia^[2]. Justifying factors include clarithromycin susceptibility to strains in patients with dyspepsia, less strain virulence (CagA negative) and differences in compliance with treatment. The lowest eradication index of *H pylori* observed in children may be due to the low prevalence of duodenal ulcer^[29]. On the other hand, patients with antral nodularity present a lower eradication rate of *H pylori* (56.2%), but lymphoid follicles are found to be associated with treatment failure in adult patients^[30]. Antral nodularity may be related to a higher inflammation intensity and more aggressive strains. However, it seems more difficult to eradicate infection with a CagA negative strain^[31]. The high incidence of side effects (67.6%), although slight and self-limited, constitutes an inconvenience for the studied regimen. The reported side effects are slight and do not compromise the success treatment (Table 3). The reported symptoms may be attributed to clarithromycin or to furazolidone. Furazolidone is a nitrofurantoin compound which has been used in the treatment of giardiasis since the 1950s. The drug has minimal adverse effects, mostly nausea, vomiting and diarrhea. Other side effects include brown discoloration of urine and hemolysis in glucose-6-phosphate dehydrogenase deficient patients and infants

younger than 1-year old^[32]. Treatment regimens with furazolidone usually present a higher incidence of side effects than traditional alternatives^[9]. Lower furazolidone doses neither affect the success treatment rate, nor decrease the frequency of adverse effects^[11].

The omeprazole dose used may be considered small in view of recent evidence that some patients need higher doses^[33]. The importance of antisecretory drugs in the eradication regimen is their direct effect on the bacterium and the better antibiotic activity at high pH^[1]. Cytochrome P2C19 is responsible for hepatic metabolism of some proton pump inhibitors, such as omeprazole, and the CYP2C19 genotype, an isoform, is associated with more rapid metabolism, constituting another risk factor for unsuccessful eradication treatment of *H pylori*^[34]. However, there are no studies describing the prevalent genotypes. Finally, some of our patients used generic omeprazole. The efficacy of *H pylori* eradication regimen with generic medication is lower than that with proprietary drugs in adult patients in Russia^[35]. Omeprazole bioavailability depends on its presentation

Most of our patients presented non-ulcer dyspepsia, a situation in which treatment of *H pylori* infection is still controversial. The treatment seems to be beneficial to some adult patients and it is estimated that 1 in 18 patients improves after the treatment^[36]. There are still important limitations in therapeutic trials for dyspepsia in children. There are no criteria for the selection of patients and no validated diagnostic and functional dyspepsia severity scores in children, which makes the generalization of results difficult. Over 50% of physicians in USA treat *H pylori* in children with dyspeptic symptoms without endoscopy^[37]. In spite of the higher symptom improvement proportion among the successfully treated patients (78.9% *vs* 50%), the study could not draw a conclusion about the clinical validity of the treatment because of the small number of studied patients (Figure 1). Other studies have reported a similar response rate in children with recurrent chronic abdominal pain^[6,38]. Long-term symptom resolution in patients with severe symptoms requiring endoscopy shows differences in epigastric pain resolution between *H pylori*-negative (3/26) and positive (7/10) patients ($P = 0.001$) after one to two years^[38]. Early clinical evaluation may underestimate the beneficial effects of the treatment and longer follow-up periods may show effective *H pylori* eradication and symptom resolution.

The tested regimen may be superior to the regimen with clarithromycin, amoxicillin and omeprazole, and can be used in the treatment of infection in patients with duodenal ulcer. Its success rate is lower in non-ulcer dyspepsia. Treatment regimens with a longer time should be tested in children.

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RAPID COMMUNICATION

Prevalence of SLC22A4, SLC22A5 and CARD15 gene mutations in Hungarian pediatric patients with Crohn's disease

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CONCLUSION: The frequency of the NOD2/CARD15 susceptibility variants in the Hungarian pediatric CD population is high and the profile differs from the adult CD patients, whereas the results for SLC22A4 and SLC22A5 mutation screening do not confirm the assumption that the carriage of these genotypes means an obligatory susceptibility to CD.

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Key words: OCTN1; OCTN2; NOD2/CARD15; Crohn's disease

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Abstract

AIM: To investigate the frequency of the common NOD2/CARD15 susceptibility variants and two functional polymorphisms of OCTN cation transporter genes in Hungarian pediatric patients with Crohn's disease (CD).

METHODS: A cohort of 19 unrelated pediatric and 55 unrelated adult patients with Crohn's disease and 49 healthy controls were studied. Genotyping of the three common CD-associated CARD15 variants (Arg702Trp, Gly908Arg and 1007finsC changes) with the SLC22A4 1672C→T, and SLC22A5 -207G→C mutations was performed by direct sequencing of the specific regions of these genes.

RESULTS: At least one CARD15 mutation was present in 52.6% of the children and in 34.5% of the adults compared to 14.3% in controls. Surprisingly, strongly different mutation profile was detected in the pediatric *versus* adult patients. While the G908R and 1007finsC variants were 18.4% and 21.1% in the pediatric group, they were 1.82% and 11.8% in the adults, and were 1.02% and 3.06% in the controls, respectively. The R702W allele was increased approximately two-fold in the adult subjects, while in the pediatric group it was only approximately 64% of the controls (9.09% in the adults, 2.63% in pediatric patients, and 4.08% in the controls). No accumulation of the OCTN variants was observed in any patient group *versus* the controls.

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract. Although the peak onset of the disease typically occurs in the second and third decades of life^[1], the incidence of pediatric cases has been strongly increasing recently^[2]. Despite the comprehensive research that has been made to discover the background of the disease, the etiology is still unknown. Besides the environmental effects, it is supposed that genetic susceptibility plays a crucial role in the development of the disease^[3,4].

Genome-wide linkage analyses have resulted in identification of several loci of potential CD susceptibility genes^[5-11]. CARD15 (NOD2) gene, which is located at the pericentromeric region of chromosome 16, was the first gene to be identified as CD gene^[12-14]. NOD2 is an intracellular protein expressed in peripheral blood monocytes, Paneth and intestinal epithelial cells; and it is important for inflammatory signal transduction *via* activation of the transcription factor, nuclear factor kappa-B (NF-κB)^[15]. Several studies on Caucasian populations have reported an association between CARD mutations and CD. Three coding variants (R702W, G908R

and 1007finsC) have been identified as independent risk factors for development of CD.

Recently two polymorphisms in the carnitine/organic cation transporter gene cluster (SLC22A4 and SLC22A5, encoding OCTN1 and OCTN2, respectively) have been found to confer risk for CD^[16]. The aim of the present study was to investigate the prevalence of these two functional variants of the SLC22A4 and SLC22A5 genes and the three CARD15 mutations in Hungarian pediatric population with CD.

MATERIALS AND METHODS

Patients

We examined 19 pediatric (14 males and 5 females; mean age: 13.4 years) and 55 adult (27 male and 28 female with real maturity onset disease; mean age: 42.3 years) patients with CD. This cohort was compared with 49 age- and sex-matched healthy controls (28 males and 21 females; mean age: 14.4 years). Both the pediatric and adult CD patients exhibited different clinical manifestations, therefore they represented mixed clinical CD populations. The diagnosis was confirmed by clinical, radiological, endoscopic and histological findings. Informed consent was obtained from each participant of the study and the study design was approved by the Local Ethics Committee.

Methods

Genomic DNA from the patients and the controls was isolated from peripheral blood using standard desalting procedure.

The presence of the NOD2 and OCTN variants was detected by direct sequencing using the primers designed in our laboratory. The primers' sequences for the PCR amplification as well as for the sequencing and annealing temperatures are listed in Table 1. The PCR was carried out in a final volume of 50 µL containing 200 µmol/L of each dNTP, 2 units of Taq polymerase, 5 µL of reaction buffer [100 mmol/L Tris HCl (pH 9.0), 500 mmol/L KCl, 15 mmol/L MgCl₂, 0.2 µmol/L of each primer and 1 µg of DNA to be amplified. The amplification was performed for a total of 35 cycles in an MJ Research PTC-200 thermal cycler. The amplification conditions were: pre-denaturation at 95°C for 2 min, denaturation at 95°C for 30 s, annealing for 30 s at the temperatures listed in Table 1 for the different SNP, primer extension at 72°C for 30 s, and the final extension at 72°C for 5 min. For DNA sequencing, a BigDye Terminator labeling was used and the analysis was performed in an ABI 3100 automatic sequencer.

Statistical analysis

Chi-square test (cross-table analysis) was used to analyze the possible associations with mutations in comparison of either the susceptibility variants and/or the normal haplotypes. $P < 0.05$ was considered statistically significant.

RESULTS

The allele frequencies are shown in Table 2. A total of

Table 1 Primer sequences and annealing temperatures for genotypings

	SNP	Primers	Tannealing (°C)
NOD2/ CARD15	R702W	F: GAGCCGCACAACCTTCAGATC R: ACTTGAGGTGCCCAACATTACG	50
	G908R	F: GTTCATGTCTAGAACACATATCAGG R: GTTCAAAGACCTTCAGAAGCTGG	50
	1007finsC	F: CCTTGAAGCTCACCATTGTATC R: GATCCTCAAAATTCTGCCATTTC	50
OCTN1	C1672T	F: AGAGAGTCTCTCTATCTGATTG R: TCCTAGCTATTCTTCCATGC	54
OCTN2	G-207C	F: AGTCCCCTGCCTTCCTAAG R: GTCACCTCGTCGTAGTCCCCG	58

Table 2 Comparison of the alleles of OCTN cation transporters and NOD2/CARD15 genes in pediatric and adult Crohn's disease patients with controls

		Pediatric patients <i>n</i> = 19 (%)	Adult patients <i>n</i> = 55 (%)	Controls children <i>n</i> = 49 (%)
CARD15 genotype				
R702W	CC	18 (94.7)	46 (83.6)	46 (93.9)
	CT	1 (5.3)	8 (14.6)	2 (4.1)
	TT	-	1 (1.8)	1 (2.0)
	T allele frequency (%)	2.63	9.09	4.08
G908R	GG	14 (73.7)	53 (96.4)	48 (98.0)
	GC	3 (15.8)	2 (3.6)	1 (2.0)
	CC	2 (10.5)	-	-
	C allele frequency (%)	18.4	1.82	1.02
1007finsC	- -	13 (68.5)	44 (80.0)	46 (93.9)
	- insC	4 (21.0)	9 (16.4)	3 (6.1)
	insC insC	2 (10.5)	2 (3.6)	-
	Cins allele frequency (%)	21.1%	11.8%	3.06%
SLC22A4 genotype				
C1672T	CC	4 (21.0)	18 (32.7)	12 (24.5)
	CT	11 (58.0)	30 (54.5)	25 (51.0)
	TT	4 (21.0)	7 (12.8)	12 (24.5)
	T allele frequency (%)	50.0	40.0	50.0
SLC22A5 genotype				
G-207C	GG	3 (15.8)	14 (25.5)	10 (20.4)
	GC	7 (36.8)	31 (56.4)	26 (53.1)
	CC	9 (47.4)	10 (18.1)	13 (26.5)
	C allele frequency (%)	65.8	46.4	53.1

52.6% of pediatric patients with Crohn's disease carried at least one NOD2 mutation compared to 34.5% of adult patients and to 14.3% of the controls (pediatric patients *vs* controls $P < 0.05$).

While the T allele frequency, leading to heterozygous and homozygous R702W mutation, was increased approximately two-fold in the adult CD population (9.09%) compared to the controls (4.08%), it was only 2.63% in the pediatric CD patients (Table 2; $P < 0.05$ comparing the pediatric susceptibility and/or normal variants *versus*

the same values of the adult patients or the controls). By contrast, the C allele frequency, encoding the G908R variant, was found highly elevated (18.4%) in pediatric patients, and was only 1.82% in adult CD patients, and 1.02% in the controls ($P < 0.05$). For the 1007finsC variant, a significantly increased prevalence was found both in pediatric (21.1%) and in adult CD (11.8%) patients as compared with the controls (3.06%) ($P < 0.05$).

There were no significant differences in the allele frequencies of SLC22A4 C1672T and SLC22A5 G-207C mutations when compared either the results of the pediatric or the adult CD populations to the results of the controls (Table 2).

DISCUSSION

The carriage rate for the three common CD-associated CARD15 mutations was reported 31% in a pediatric CD population in North America^[17], 60% in Germany^[18], 51.5% in the Israeli Jewish patients^[19] and 40.7% in an Italian cohort^[20]. In two studies, the cytosine insertion mutation 3020insC was significantly more common in the pediatric CD population^[18,21], whereas among the Jewish patients G908R missense mutation was the most frequent variant^[19,22].

An association of the three CARD15 mutations R702W, G908R and 1007finsC with CD has been confirmed in several studies^[17,18,23], although different allele frequencies have been observed. While the allele frequencies of the three mutations were almost the same (8.3%, 8.3% and 7.4%) in the Italian pediatric patients^[20], the G908R variant was the most frequent among Jewish children^[19] and 1007finsC was more common in Germany^[18] and in the USA^[21]. An earlier onset of disease was found in the presence of a CARD15 mutation in three additional studies^[23-25]. These findings suggest that CARD15 mutations may be more frequent in pediatric CD.

To our surprise, in our study groups, two mutations, G908R and 1007finsC, were significantly more frequent in the pediatric population with the allele frequencies of 18.42% in children *versus* 1.02% in controls and of 21.05% in children *versus* 3.06% in controls, respectively. The genotyping results for the adult population are in agreement with previous Hungarian findings^[26,27].

The OCTN1 and OCTN2 transporters mediate the transport of carnitine and a wide range of organic cations^[28-30] and have an important role in the energy supply of epithelial cells. Recently, it has become clear that the OCTN1 also transports the ergothioneine^[31], and the affinity parameters make it almost unquestionable that the carnitine transport function is secondary. A C1672T missense substitution in exon 9 of the SLC22A4 gene results in marked changes in OCTN1 transporter activity, whereas G-207C transversion in the SLC22A5 promoter region causes OCTN2 promoter function impairment.

By resequencing the five genes in the IBD5 interval, which harbors the cytokine gene cluster, and, therefore, is an attractive candidate region for IBD, Peltekova *et al*^[16] identified 2 novel polymorphisms in the SLC22A4 and SLC22A5 genes. These two mutations (SLC22A4 C1672T and SLC22A5 G-207C) form a two-allele risk haplotype

(OCTN-TC) which was associated with CD and showed significant interactions with CD-associated CARD15 mutations. This observation has been repeatedly confirmed^[32-34], although, in the absence of the IBD5 risk haplotype, no association of OCTN1/2 variants with CD was reported in two studies^[33,34]. While a Belgian group^[35] found that the OCTN did not play a role in the susceptibility to CD, the two functional variants in the SLC22A4 and SLC22A5 genes were completely absent in Japanese^[36].

In our study, which is probably the first in the international literature for pediatric population, we could not find accumulation of any of the susceptibility haplotypes either in the pediatric or in the adult CD subjects, thereby not supporting the susceptibility role of the above haplotypes in the development of CD.

In conclusion, we observed an accumulation of CARD15 mutations in pediatric cases, whereas the results for SLC22A4 and SLC22A5 mutation screening do not confirm the assumption that the carriage of these genotypes means significant susceptibility to CD. However, for genotype-phenotype correlations, further studies are needed with larger study populations.

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RAPID COMMUNICATION

Efficacy of low dose peginterferon alpha-2b with ribavirin on chronic hepatitis C

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Abstract

AIM: To assess the efficacy of peginterferon alpha 2b at doses of 50 µg weekly and 80 µg weekly (based on body weight) plus ribavirin in HCV genotype 2 and genotype 3 chronic hepatitis C patients.

METHODS: During the study period of Jan 2002 to Dec 2003, all patients diagnosed as chronic hepatitis C or HCV related compensated cirrhosis were treated with peginterferon alpha 2b 50 µg S/C weekly (body weight < 60 kg) or 80 µg S/C weekly (body weight > 60 kg) plus ribavirin 800 mg/d for 24 wk.

RESULTS: Overall 28 patients, 14 patients in each group (based on body weight) were treated during the period. Out of 28 patients, 75% were genotype 3, 18% were genotype 2 and 7% were genotype 1. The mean dose of peginterferon alpha 2b was 0.91 µg/kg in group 1 and 1.23 µg/kg in group 2 respectively. The end of treatment and sustained virologic response rates were 82% and 78% respectively. Serious adverse effects were seen in 3.5% patients.

CONCLUSION: Low dose peginterferon alpha 2b in combination with ribavirin for 24 wk is effective in HCV genotype 2 and 3 chronic hepatitis C patients.

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Key words: Chronic hepatitis C; Peginterferon alpha 2b; Ribavirin

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INTRODUCTION

The hepatitis C virus is a major cause of liver diseases affecting 170 million people worldwide^[1]. In India, the estimated prevalence of hepatitis C virus infection is 1.8%. Genotypes 2 and 3 are predominant in Indian population^[2].

Therapy for chronic hepatitis C has greatly improved over the last decade and is still evolving^[3-8]. The introduction of pegylated form of interferons and addition of ribavirin have further improved the efficacy of therapy in chronic hepatitis C. Higher sustained virological response (SVR) rates in patients with chronic hepatitis C have been reported with pegylated form of interferons compared with standard interferons both as monotherapy as well as in combination with ribavirin^[9-13].

Peginterferon alpha-2b is recommended at a dose of 1.5 µg/kg body weight in combination with ribavirin for treatment of chronic hepatitis C. An earlier study has reported similar efficacy of peginterferon alpha-2b at doses of 1 µg/kg and 1.5 µg/kg body weight in treatment of chronic hepatitis C as monotherapy^[9]. Recent studies have shown that 82%-90% SVR could be achieved in HCV genotype 2 and 3 patients using peginterferon and ribavirin combination^[13,14].

Interferon-based regimens are associated with significant side effects and costs. The cost of therapy in developing countries is a major limiting factor in initiating treatment. There is limited data on efficacy of low dose peginterferon alpha 2b plus ribavirin on chronic hepatitis C. It would be worthwhile to study whether the dose of peginterferon alpha-2b could be lowered from 1.5 µg/kg body weight to 1 µg/kg body weight in combination with standard dose of ribavirin without compromising the efficacy of therapy in HCV genotype 2 and 3 chronic hepatitis C patients.

Therefore, we conducted a non-randomized pilot study to assess the efficacy of peginterferon alpha-2b, at doses of 50 µg and 80 µg (based on body weight) plus ribavirin at standard dose in HCV genotype 2 and 3 related chronic hepatitis and compensated cirrhotic patients.

MATERIALS AND METHODS

Subjects

All patients with chronic liver disease attending our hospital from Jan 2002 to Dec 2003 were evaluated for HCV infection. After detailed history and clinical examination, patients underwent routine hematological investigations, liver function test, abdominal ultrasound

and upper GI endoscopy. All patients were tested for the presence of anti-HCV antibodies with Abbott diagnostic test kit according to the manufacturer's instructions. Those patients who tested positive for anti-HCV were further investigated for HCV RNA. HCV RNA was tested by RT-PCR (SDS) using 30 base pair long dual labeled oligonucleotide TaqMan probe (Rotorgene from Corbett research Australia). HCV genotype was tested by molecular based linear array system using COBAS AMPLICOR.

All patients diagnosed with chronic hepatitis C and compensated cirrhosis were eligible for study. Patients meeting with the following criteria were excluded: Presence of decompensated cirrhosis, hepatitis B coinfection, HIV co-infection, renal failure, concomitant malignancy, co-morbid serious cardiac and respiratory diseases, neuropsychiatric disorders, pregnancy, lactating mothers and alcohol abuse.

Study protocol

All eligible patients who gave informed consent were included in the study. Patients were treated with either 50 µg or 80 µg subcutaneous (s/c) weekly dose of pegylated interferon alpha 2b based on body weight plus 800 mg daily dose of ribavirin. Those patients who weighed less than 60 kg were administered a dose of 50 µg per week of pegylated interferon alpha 2b while those weighing more than 60 kg received 80 µg S/C per week. All patients were evaluated as outpatients weekly for 4 wk, then at wk 8, 12, 16 and 24 during treatment. Following the completion of treatment, patients were evaluated at wk 4, 12 and 24. On each visit during follow-up, routine hematological workup was done. Besides, relevant investigations were performed as and when necessary. Qualitative HCV RNA was tested after 12 wk of treatment, at end of treatment (24 wk) and 6 mo after completion of treatment. Liver biopsy was not considered mandatory in study protocol and was done in those patients who agreed to undergo the procedure. Side effects of the therapy were carefully recorded during follow-up. Sustained virologic response was defined as normalization of ALT and negative HCV-RNA 6 mo after completion of therapy.

Informed, written consent was taken from all the patients. Our hospital ethics committee approved the study protocol.

Statistical analysis

The quantitative values were expressed as mean \pm SD. Fisher's exact test was used for statistical comparison of the data. $P < 0.05$ was considered as significant.

RESULTS

Overall 28 patients (25 males and 3 females) were included in the study. The demographic profile and clinical characteristics of the patients are shown in Table 1.

Fifteen out of 28 patients (54%) were chronic hepatitis and the remaining 13 (46%) were compensated cirrhosis (Child A). Eighteen (64%) of 28 patients had elevated ALT > 1.5 times the upper limit. The pretreatment ALT levels were $89 \text{ IU/L} \pm 28 \text{ IU/L}$. Out of 28 patients, 75%, 18% and 7% were genotype 3, 2 and 1 respectively. Fourteen

Table 1 Demographic, clinical, biochemical and molecular profiles of patients (mean \pm SD, $n = 28$)

Demographic profile	
M/F	25:3
Age (yr)	47.5 ± 13.2
Body weight (kg)	60.6 ± 9.18
Clinical profile	
Chronic hepatitis C (n)	15
Compensated cirrhosis (n)	13
ALT ($> 40 \text{ IU/L}$)	18
ALT ($< 40 \text{ IU/L}$)	10
Molecular profile	
Genotype 3 (n)	21
Genotype 2 (n)	5
Genotype 1 (n)	2
Follow-up (mo)	18.1 ± 6.7

patients (mass $< 60 \text{ kg}$) were administered 50 µg S/C weekly dose of peginterferon alpha 2b plus 800 mg oral daily dose of ribavirin. The mean dose of peginterferon alpha 2b was 0.91 µg/kg body weight ($0.6 \text{ µg/kg} \pm 1.22 \text{ µg/kg}$) in this group.

The second group of 14 patients (mass $> 60 \text{ kg}$) received 80 µg S/C weekly dose of peginterferon alpha 2b with daily oral dose of 800 mg ribavirin. The mean dose of peginterferon alpha 2b in this group was 1.23 µg/kg ($1.03 \text{ µg/kg} \pm 1.33 \text{ µg/kg}$). One patient discontinued treatment due to severe side effects and another patient lost to follow up after 2 wk. Twenty-six out of 28 patients completed the 24 wk treatment. At the end of 24 wk treatment, 23 patients were negative for HCV RNA and 3 patients tested positive for HCV RNA. On further follow up, one patient tested positive for HCV RNA 6 mo after completion of therapy. Overall, at the end of treatment, 23 (82%) of 28 patients showed response to therapy. The SVR was achieved in 22 (78%) out of 28 patients. Though the dose per kg body weight of peginterferon alpha 2b was significantly different in two groups ($P < 0.002$), there was no difference in response to therapy with respect to dose or genotype (Table 2). During the follow-up of $18.1 \pm 6.7 \text{ mo}$, one non-responder patient developed hepatocarcinoma (HCC) and died of liver failure following variceal bleeding.

One patient (3.5%) developed unbearable weakness and burning in urethra and discontinued the treatment. Seven (25%) of 28 patients developed mild leucopenia and thrombocytopenia which required temporary interruption of treatment for 1-2 wk. However, none of these 7 patients discontinued treatment any more and completed the 24 wk treatment.

DISCUSSION

The combination of peginterferon and ribavirin is the mainstay of treatment in chronic hepatitis C. Both forms of peginterferon alpha 2a and alpha 2b in combination with ribavirin have shown SVR rates from 79%-93% in genotype 2 and 3 chronic hepatitis C patients, making it a potentially curable disease^[14,15]. In our study, end of treatment response (ETR) and SVR rates were 82%

Table 2 Response of therapy with respect to dose and genotype

Patients (n = 28)	Response to peginterferon alpha 2b Dose 50 mcg (n = 14)		Response to peginterferon alpha 2b Dose 80 mcg (n = 12) ¹	
	Yes	No	Yes	No
Genotype 1 (n = 2)	-	1	1	-
Genotype 2 (n = 5)	3	-	2	-
Genotype 3 (n = 21)	8	2	8	1

¹Two patients in group 2 did not complete therapy.

and 78% respectively. The mean dose per kg body weight of peginterferon alpha 2b was 0.9 µg/kg and 1.23 µg/kg in the two groups, respectively. Lindsay *et al*^[9] have shown similar SVR rates with 1.5 µg/kg and 1 µg/kg of peginterferon alpha 2b as monotherapy.

In a recent study, Zeuzem *et al*^[14] have shown SVR rates of 79% and 93% in genotype 3 and genotype 2 chronic hepatitis C patients respectively when treated with peginterferon alpha 2b 1.5 µg/kg S/C weekly plus ribavirin 800-1400 mg/d based on body weight. In the same study, higher virologic response rate was observed in HCV genotype 3 patients with baseline HCV RNA concentration of < 600 IU/L compared to those with baseline HCV RNA > 600 IU/L (85% *vs* 59%). In our study, quantitative HCV RNA assay was not included in the study protocol, hence data could not be analysed based on HCV RNA levels. However, our results are comparable with those reported by Zeuzem *et al*^[14], albeit at a much lower dose. One patient (3.5%) reported serious side effects resulting in discontinuation of treatment. The remaining patients tolerated the treatment well. The overall safety profile was much improved compared with earlier studies^[16]. One patient who was a non-responder to treatment died during follow-up due to HCC related decompensation, suggestive of progressive disease.

HCV genotypes 1b and 2a are common in China^[15]. In Japan, genotypes 1b, 2a and 2b are prevalent. Genotype 3 is also observed in Southeast Asian countries. In India, genotypes 2 and 3 are predominant HCV genotypes^[2]. These genotypes 2 and 3 respond well to treatment^[11,13,14]. But the cost of the treatment is a major limiting factor in developing countries. Our study has shown that > 80% SVR rates could be achieved in HCV genotype 2 and 3 infected chronic hepatitis C patients with lower than recommended dose of pegylated interferon alpha 2b. In light of encouraging results of our study, a randomized controlled trial is needed to compare the efficacy of lower doses of pegylated interferon alpha 2b, ie, with 1.5 µg/kg body weight of peginterferon alpha 2b in combination with standard dose of ribavirin with specific reference to Asian population.

In conclusion, the present study shows that lower than recommended dose of peginterferon alpha 2b in combination with ribavirin for 24 wk is effective in HCV genotype 2 and 3 chronic hepatitis C patients.

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Enhanced expression of epidermal growth factor receptor gene in gastric mucosal cells by the serum derived from rats treated with electroacupuncture at stomach meridian acupoints

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Abstract

AIM: To investigate the effect of serum derived from rats treated with electroacupuncture at stomach meridian acupoints on the expression of epidermal growth factor receptor (EGFR) gene in gastric mucosal cells.

METHODS: The stress-induced gastric mucosal injury in rat model was established by water-immersion and restrained stress methods. 52 rats were randomly divided into: normal group ($n = 8$), model group ($n = 8$), model serum group ($n = 12$), stomach serum group ($n = 12$), and gallbladder serum group ($n = 12$). The gastric mucosal cells were separated by pronase-EDTA digestion method and incubated with serum. The EGFR gene expression in gastric mucosal cells was detected by reverse transcription-polymerase chain reaction (RT-PCR) method.

RESULTS: Compared with normal group (0.6860 ± 0.0594), the serum derived from rats of the stomach group (1.2272 ± 0.0813 , $P = 0.00 < 0.01$) and gallbladder group (0.9640 ± 0.0387 , $P = 0.00 < 0.01$) had a tendency to enhance the EGFR gene expression in gastric mucosal cells. Such tendency existed in the model group (0.7104 ± 0.0457) but with no significant difference ($P = 0.495 > 0.05$) and in model serum group (0.8516 ± 0.0409) with an extremely obvious difference ($P = 0.001 < 0.01$). Furthermore, the EGFR gene expression in stomach serum group was significantly higher than that in gallbladder serum group ($P = 0.00 < 0.01$).

CONCLUSION: The present study shows that serum

derived from rats treated with electroacupuncture at stomach meridian acupoints can distinctly increase the EGFR gene expression of gastric mucosal cells. Therefore, there is certain meridian specificity in the serum, which could provide a proof for the TCM theory "particular relation between meridian and internal organ".

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Key words: Electroacupuncture; Serum; Stomach meridian acupoints; Gastric mucosal cells; Epidermal growth factor receptor; Gene expression

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INTRODUCTION

Gastric mucosal damage is a common pathological reaction in the diseases of the digestive system. The acupuncture and moxibustion are very effective cure for this damage^[1,2]. Previous experimental studies demonstrated that epidermal growth factor (EGF) and transforming growth factor- α (TGF- α) were the most important peptides for the repair of the gastric mucosal injury^[3]. Acupuncture at gastric meridian acupoint could alter gastric motility and secretion and also the content of gastrin, substance P, EGF and TGF- α in serum and gastric mucosa^[4,5]. Recent research indicated that EGFR was closely related to the healing of impaired gastric mucosa, which was of great importance to the gastric mucosal protection and repair after damage^[6]. The EGFR belongs to the family of trans-membrane tyrosine protein kinase (TPK). Activation of EGFR stimulates cell proliferation, differentiation, adhesion, and migration^[7,8]. The aim of this study was to examine the effect of serum derived from rats treated with electroacupuncture at stomach meridian acupoints on the expression of EGFR gene in gastric mucosal cells. This would hopefully clarify the humoral

mechanism of acupuncture effect on gastric mucosal cells and the essential correlation of the meridian acupoints and internal organs.

MATERIALS AND METHODS

Reagents

Pronase and dithiothreitol (DTT) were purchased from MERK. Bovine serum albumin (BSA) was obtained from Biosharp. Percoll was purchased from Pharmacia, Dulbecco's Modified Eagle Medium (DMEM) from Hyclone, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) from Biosource, Trizol reagent was obtained from Invitrogen. AMV reverse transcriptase, ribonuclease inhibitor (RNasin), dNTPs, Taq DNA polymerase, 100 bp DNA ladder, diethylpyrocarbonate (DEPC), oligodT18 primer, and gelose were purchased from Promega. Tyrosine kinase inhibitor (PD153035) was purchased from Calbiochem. EGFR and the internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Invitrogen. All other reagents were analytically pure.

The stress-induced gastric mucosal injury in rats

Water-immersion and restrained stress methods were adopted^[9]. Before modelling, the experimental rats were fasted for 24 h and had free access to water only. Rats were fixed on boards and were immersed vertically in a homeostatic bath at $23 \pm 1^\circ\text{C}$ for 10 h, with the liquid surface up to the level of the xiphoid process of the sternum.

Experimental design

Sprague-Dawley (SD) male and female rats, with an average weight of 200 ± 30 gm, were supplied by the Experimental Animal Center at Hunan Agriculture University (Permission number: 20030316) 2 wk before the experiment. During this period, they had access to Purina rat chow and water. Animals were fasted overnight before the experiments. Fifty-two rats were randomly divided into normal group, model group, model serum group, stomach serum group and gallbladder serum group. 8 rats were in the normal group and model group. Each of the model serum group, stomach serum group and gallbladder serum group included 12 rats.

Four rats of each of the model serum group, stomach serum group, and gallbladder serum group were selected at random for deriving serum, and the remaining 8 rats were used for isolating gastric mucosal cells. Acupoints location was defined by reference of rat-acupoint-atlas and analogy to human body^[10]. According to the induction stated above, three pairs of acupoints consisting of Sibai (ST 2), Liangmen (ST 21), and Zusanli (ST36) in the stomach Meridian, were designed, which represent acupoints of different level (head, trunk, and limb). Also, 3 pairs of acupoints of the gallbladder Meridian in the same horizontal level were selected: Yangbai (GB 14), Riyue (GB24), and Yanglingquan (GB 34).

Pairs of stainless-steel needles of 0.25 mm in diameter were inserted into the acupoints stated above in experimental rats. The needles were connected to

the output of an electronic pulse generator, a medical electroacupuncture stimulator (Model G6805-1, made by Shanghai Medical Electro-apparatus Factory, China), which achieves intermittent-and-irregular wave (intermittent wave: 4 Hz, irregular wave: 20 Hz), constant time of 30 min per day, ten days, while there was a light vibration in the lower limbs of rats.

Isolation of gastric mucosal cells

Animals were fasted overnight before the experiments. All experiments were performed using freshly isolated gastric mucosal cells. The contents of the stomach were washed out with phosphate-buffered saline (PBS). The stomach was then ligated at the base of the forestomach and the proximal end of the antrum to obtain mucosal cells primarily from the oxyntic region. After being transformed into inside-out gastric bags, they were filled with 2.5 mL of 1 mg/mL pronase solution in buffer A (0.5 mmol/L NaH_2PO_4 , 1.0 mmol/L Na_2HPO_4 , 20 mmol/L NaHCO_3 , 80 mmol/L NaCl, 5.0 mmol/L KCl, 50 mmol/L HEPES, 11 mmol/L glucose, 0.02 mmol/L BSA, 2 mmol/L EDTA, pH 7.4). The filled gastric bags were incubated in pronase-free buffer A at 37°C for 30 min. The gastric bags were then transferred into buffer B (0.5 mmol/L NaH_2PO_4 , 1.0 mmol/L Na_2HPO_4 , 20 mmol/L NaHCO_3 , 80 mmol/L NaCl, 5.0 mmol/L KCl, 50 mmol/L HEPES, 11 mmol/L glucose, 0.01 mmol/L BSA, 1 mmol/L CaCl, 1.5 mmol/L MgCl, pH7.4) and gently agitated by a magnetic stirrer at room temperature for 1h. The gastric mucosal cells dispersed in buffer B were collected by centrifuging at 3000 rpm for 5 min and subsequently resuspended in serum-free DMEM^[11,12].

Serum collection

The blood was sampled from carotid artery after rats were treated according to the requirement of experimental procedures. Then the blood was transferred into centrifuge tubes and placed steadily for 2 h at 37°C . Tubes were centrifuged at 2500 rpm for 10 min. The serum was carefully sucked and frozen at -20°C . The gastric mucosal cells were incubated with 100 mL/L serum at 37°C for 30 min in the experiment^[13,14].

RNA extraction

Following the treatment stated above, gastric mucosal cells obtained from each rat were collected in Eppendorf tubes and kept in the -80°C . Eight samples from each group were selected randomly for RNA extraction. Total RNA was isolated from samples of gastric mucosal cells by using a guanidium isothiocyanate/phenol chloroform single step extraction kit from Stratagene. Total RNA was precipitated in ethanol and resuspended in sterile RNAase-free water for storage at -80°C until use. Total RNA was quantified spectrometrically at 260 nm, and the quality of isolated RNA was analyzed on agarose gels under standard conditions.

Reverse transcription reaction

Total RNA (10 μL , about 0.5 μg /sample) was reverse transcribed (RT) using oligo (dT) 18 primers 1 μL , $5 \times$

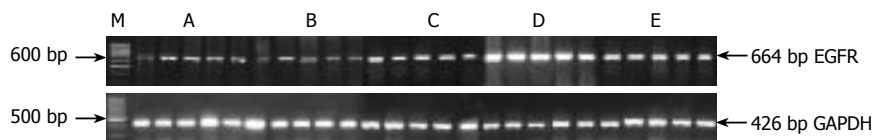


Figure 1 Electrophoresis of EGFR mRNA and GAPDH mRNA RT-PCR product in gastric mucosal cells. M: Marker; A: Normal group; B: Model group; C: Model serum group; D: Stomach serum group; E: Gallbladder serum group.

Table 1 The primer sequences and sizes of amplification products

EGFR	Forward primer: 5'-AGT GGT CCT TGG AAA CTT GG-3'	664 bp
	Reverse primer: 5'-GTT GAC ATC CAT CTG GTA CG-3'	
GADPH	Forward primer: 5'-TGC TGA GTA TGT CGT GGA GTC -3'	426 bp
	Reverse primer: 5'-AAG GCC ATG CCA GTG AGC TTC -3'	

Table 2 The EGFR gene expression in gastric mucosal cells (mean \pm SD, $n = 8$)

Group	EGFR mRNA/GAPDH mRNA
Normal group	0.6860 \pm 0.0594
Model group	0.7104 \pm 0.0457 ^d
Model serum group	0.8516 \pm 0.0409 ^{b,d}
Stomach serum group	1.2272 \pm 0.0813 ^b
Gallbladder serum group	0.9640 \pm 0.0387 ^{b,d}

^b $P < 0.01$ vs Model group; ^d $P < 0.01$ vs Stomach serum group.

RT-buffer 4 μ L, dNTPs (10 mmol/L) 1 μ L, RNasin (20 MU/ μ L) 0.5 μ L, M-MULV reverse transcriptase (200 MU/ μ L) 1 μ L, and DEPC-treated water 2.5 μ L in a 20 μ L reverse transcription reaction system. The reaction was performed at 42°C for 30-60 min so that target mRNA was transcribed into cDNA. The tubes were cooled and centrifuged for several seconds.

Polymerase chain reaction (PCR)

An aliquot of the RT product of each sample (1/20 of the total volume) was used in the PCR amplification reactions for EGFR and GAPDH. The PCR reaction contained 4 μ L cDNA, 10 \times PCR buffer 5 μ L, dNTPS (10 mmol/L) 1 μ L, oligonucleotide primers sense/antisense (10 mmol/L) 1 μ L (primer sequences are stated below), Taqase 1 μ L, ddH₂O 32 μ L in a total volume of 50 μ L. Reaction mixtures were incubated for predenaturation at 94°C for 2 min, followed by 38 cycles for EGFR (denaturation at 94°C for 30 s, annealing at 53°C for 1 min, and extension at 72°C for 1 min) and 25 cycles for GAPDH (denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s), and a final extension at 72°C for 5 min.

Primer design and the RT-PCR product electrophoresis

To use of the relatively quantitative method to measure EGFR gene expression, rat GAPDH was selected as internal control substance. The primer sequences and sizes of amplification products are as shown in Table 1. Five microliter PCR products were analyzed on 10 g/L agarose gel containing ethidiumbromide with TBE buffer at 80 V for 40 min and photographed under UV illumination. The band intensities were quantified by densitometry. EGFR and GAPDH PCR products were, respectively, 664 and 426 base pairs (Table 1). EGFR and GAPDH were determined by computer-assisted densitometric scanning. Signals were quantified by density analysis of the digital images using Eagle Eye II image software (Stratagene) and EGFR/GAPDH quotient indicated the relative expression of EGFR. Experiments were performed in triplicate.

Statistical analysis

The data for each group were expressed as mean \pm SD.

Comparison between groups was assessed using one-way analysis of variance (ANOVA). Differences were considered statistically significant if the P value was less than 0.05. Software SPSS 13.0 was used in all statistical tests.

RESULTS

Expression of EGFR gene in gastric mucosal cells

By using RT-PCR, the EGFR gene expression in gastric mucosal cells were detected in rats of normal group and model group as a weak signal but it was well-defined among other groups: model serum group, stomach serum group and gallbladder serum group. Compared with Model serum group, the serum in stomach serum group and gallbladder serum group appeared to up-regulate significantly the EGFR gene expression in gastric mucosal cells, $P < 0.01$, and obvious difference between stomach serum group and gallbladder serum group was found ($P < 0.01$). However, there was no difference between normal group and model group, $P > 0.05$ (Table 2; Figure 1).

DISCUSSION

According to the classical Traditional Chinese Medicine (TCM) theory, there is a particular relation between meridian acupoints and viscera and the functional activities of the organism can be regulated by acupuncture at the meridian acupoints. However, it is still unknown how the acupuncture regulates the functional activities of the organism, and what is essential for the relationship between meridian acupoints and viscera. The present study proved that the acupuncture at the stomach meridian acupoints could improve gastric mucosal protection mechanism and that it is a very effective cure for gastrointestinal diseases^[15,16]. Acupuncture at acupoints of Sibai (ST2), Liangmen (ST21), and Zusanli (ST36), could produce certain ameliorative effect through the following mechanisms: augmentation of the gastric antrum, reinforcement of pressure on gastric pyloric sphincter, stimulation or inhibition of related gastrointestinal peptide secretion^[17,18]. All of these have provided experimental

evidence for the theory of "Particular relationship between gastric meridian and the stomach". However, the functional mechanism of the repair of gastric mucosal lesion is not entirely clear, and the humoral factor of acupuncture and moxibustion effect is still unknown.

The mucosal lining of the gastrointestinal tract, especially the stomach, is easily exposed to a variety of exogenous injurious agents, including non-steroidal anti-inflammatory drugs and ethanol. Each of these agents either alone or in combination with others may induce mucosal injury. However, a number of *in vivo* and *in vitro* studies have demonstrated that the gastric mucosa of animals possesses the inherent capacity to repair after mild injury^[19]. The cellular protective functions against damage maybe accomplished in several ways. There are evidences for participation of both the early phase of epithelial repair, known as restitution, marked by increased cell migration but no proliferation, and the delayed phase of cell renewal, marked by proliferation, differentiation and migration^[20,21].

In general, EGFR is one of the recently described members of cell membrane proteins. It is made of 1186 amino acids. As a trans-membrane receptor of tyrosine protein kinase family, EGFR plays a very important role in regulating healing process of damaged gastric mucosa, and regulates cell metabolism, proliferation, differentiation, migration and other biological phenomena. Many studies indicated that there was an elevated EGFR expression during the healing course of damaged gastric mucosa. Therefore, EGFR is of a great importance to the gastric mucosal protection and injury healing^[22,23]. The relationship between EGFR and its downstream signal transduction pathway and the healing of gastric mucosal injury is increasingly becoming a focus of researchers' attention. This study assessed, by RT-PCR methods, the EGFR mRNA expression in gastric mucosal cells of the rat after incubation with 10% serum for 30 min. The data showed that EGFR mRNA expression in gastric mucosal cells was enhanced shortly after incubation with the serum derived from the rats. Meanwhile, it was proved that the serum derived from the rats treated with electroacupuncture had an obvious tendency to stimulate the EGFR mRNA expression in gastric mucosal cells. In addition, EGFR mRNA expression in stomach serum group was much higher than that in model serum group and gallbladder serum group. Therefore, we hypothesize that the serum derived from rats treated with electroacupuncture contains many kinds of active substances that stimulated the EGFR gene in the gastric mucosal cells. This study also indicated that the discrepancy in the expression of EGFR gene may be the underlying mechanism of different effect of electroacupuncture at acupoints of gastric meridian and that of gallbladder meridian. Thus, this could be a proof for the TCM theory "particular relation between SMFY and the stomach". The active substance (s) in the serum derived from the rats treated with electroacupuncture at stomach meridian acupoints is (are) unknown, and therefore, more research using proteomic technology is needed.

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CASE REPORT

Percutaneous transarterial embolization of extrahepatic arteroportal fistula

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Abstract

Arteroportal fistula is a rare cause of prehepatic portal hypertension. A 44-year-old male with hepatitis virus C infection was admitted for acute variceal bleeding. Endoscopy showed the presence of large esophageal varices. The ultrasound revealed a mass near the head of pancreas, which was characterized at the color-Doppler by a turbulent flow, and arterialization of portal vein flow. CT scan of abdomen showed a large aneurysm of the gastroduodenal artery communicating into the superior mesenteric vein. The sinusoidal portal pressure measured as hepatic vein pressure gradient was normal, confirming the pre-hepatic origin of portal hypertension. The diagnosis of extrahepatic portal hypertension secondary to arteroportal fistula was established, and the percutaneous embolization was performed. Three months later, the endoscopy showed absence of esophageal varices and ascites. At the moment, the patient is in good clinical condition, without signs of portal hypertension.

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Key words: Portal hypertension; Arteroportal fistula; Portal shunt; Esophageal varices bleeding; Embolization; Interventional radiology; Pseudoaneurysm

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INTRODUCTION

Extrahepatic arteroportal fistula is a rare disorder

of hepatic vasculature characterized by anomalous communication between arteries and portal vein system most commonly caused by abdominal trauma, and iatrogenic damage induced by procedures such as liver biopsies, tumors and congenital vascular malformations^[1]. Arteroportal fistula may cause severe portal hypertension which leads to gastroesophageal variceal bleeding, refractory ascites, diarrhea, and hepatic encephalopathy. The recent progress in computed tomography and magnetic resonance technology with no invasive angiography imaging, makes it possible to confirm the suspicious arteroportal fistula at the Doppler ultrasonography and guide the choice of the treatment. The hepatic vein portal pressure gradient (HVPG) measurement may verify the diagnosis of extrahepatic portal hypertension.

CASE REPORT

A 44-year-old male was admitted to our Institution because of acute variceal bleeding and a clinical diagnosis of cirrhosis. The patient underwent a total gastrectomy in 1984 for gastroduodenal ulcer. In December 2000, there was a biopsy-proven chronic active hepatitis secondary to hepatitis virus C infection; and a long-term biochemical and virological response was obtained with a 12-mo course of combined antiviral treatment with PEG-IFN plus Ribavirin. Upon admission, the liver function tests were made and the results were: AST 34 U/L, ALT 61 U/L, INR 1.2, PT 63%, serum albumin 33 g/L, serum bilirubin 1.1 mg/dL, and platelets count $15 \times 10^3 \mu\text{L}$.

The endoscopy showed the presence of large esophageal varices with cherry red spots, localized in the medial and lower third of the esophagus and extended into the subcardial tract; the varix actively bleeding was treated with banding legations. Two days later, the ultrasonography showed homogeneous liver texture, enlarged spleen (a longitudinal diameter of 16.3 cm) and absence of ascites. However, an anechoic mass 2.5 cm in diameter was detected next to the head of the pancreas. The color-Doppler demonstrated turbulent flow, and arterialization of portal vein flow, with an increased peak velocity (83 cm/sec) (Figure 1). All these findings were suggestive for extrahepatic arteroportal fistula. A 16 slices-MDCT scan of the abdomen was performed, using 150 mL of isosmolate iodate contrast media (1.8 mL/kg), injection-velocity of 5 mL/sec, pitch 1, thickness of 2.5 mm, interval reconstruction of 0.6 mm, and triple-phase acquisition. Maximum Intensity Projection

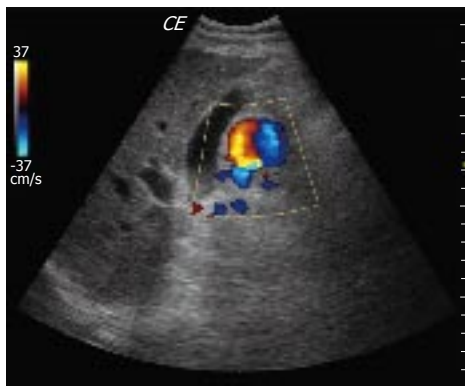


Figure 1 Color-Doppler US showed an anechoic mass, 2.5 cm in diameter, next to the head of the pancreas with turbulent flow inside.

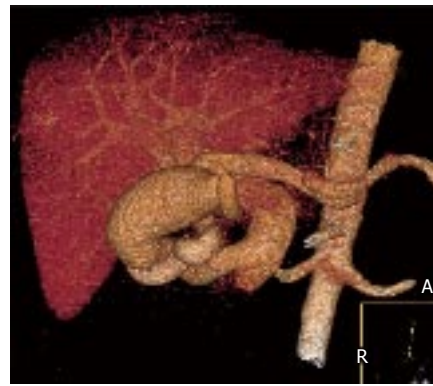


Figure 2 MDCT 3D reconstruction showed the presence of a large aneurysm filled with a dilated gastroduodenal artery and draining into the superior mesenteric vein.



Figure 3 Celiac axis arteriogram confirmed a large aneurysm of gastro-duodenal artery and a fistulous communication with the main portal vein.



Figure 4 Arteriogram performed after coils embolization showed complete occlusion of the fistula.

(MIP) and Volume Rendering (VR) 3D angiography reconstructions clearly confirmed the presence of a large aneurysm filled by a dilated gastroduodenal artery, draining into the superior mesenteric vein (Figure 2). Other CT findings were normal liver, presence of esophageal varices and splenomegaly (volume of 910 mL). The HVPG was normal (5 mmHg), confirming the extrahepatic origin of portal hypertension. The diagnosis of extrahepatic portal hypertension secondary to arteroportal fistula was established. However, the patient refused the percutaneous arterial embolization of the fistula.

Seven months later a clinical examination revealed the presence of ascites. The MRI confirmed the presence of free fluid into the abdomen, bilateral pleural effusion and no changes in the aneurysm. The percutaneous embolization was then accepted. The initial angiogram of the celiac axis confirmed the large aneurysm of the gastroduodenal artery and a fistulous communication with the main portal vein (Figure 3). After selective catheterization of the aneurysm, several metallic coils (8 mm in diameter) were placed in both the aneurysm and the fistula. The post-procedure arteriogram showed the complete occlusion of the fistula (Figure 4).

One month later, the endoscopy demonstrated the absence of esophageal varices. Three months later, a CT scan showed a complete thrombosis of the arteroportal fistula, the absence of ascites and pleural effusion, and a significant reduction of the spleen volume (689 mL). Now, the patient is free of symptoms with normal liver function

tests: AST 34 U/L, ALT 32 U/L, serum bilirubin 0.8 mg/dL, albumin 3.9 g/dL, PT 98%, INR 0.8, and platelets count $22 \times 10^3 \mu\text{L}$.

DISCUSSION

The arteroportal fistula is a rare disorder of hepatic vasculature characterized by anomalous communication between the arteries and portal venous system. Extrahepatic arteroportal fistulas are less common than the intrahepatic ones. The most common causes of arteroportal venous fistula are traumas, iatrogenic damage and congenital vascular malformations^[1]. In our patient there was no history of traumas or other vascular malformations, suggesting that the previous gastrectomy might be the most likely cause of arteroportal fistula. It is known that this vascular disorder may cause portal hypertension in non-cirrhotic liver^[2,3], due to an increased arterial blood flow. In addition, the nodular regenerative hyperplasia and hepatoportal sclerosis with fibrosis may develop during the follow-up period and worsen portal hypertension^[3].

In our case, the arteroportal venous fistula was well detected by Doppler ultrasonography, and confirmed by computed tomography and magnetic resonance angiography.

Severe portal hypertension with large esophageal varices, ascites and pleural effusion was maintained by the increased portal venous inflow. This is confirmed by a

normal value of HVPG that rules out the hepatic origin of portal hypertension. The arteroportal venous fistula can be treated with percutaneous arterial occlusion^[4]. Notably, esophageal varices and ascites disappeared after the embolization, which further confirmed the role of increased portal blood inflow.

In conclusion, arteroportal fistula is a rare cause of portal hypertension in non-cirrhotic liver and may cause variceal bleeding and ascites. Imaging studies are useful to detect it and the HVPG measurement is useful in confirming the extrahepatic origin of portal hypertension. The percutaneous arterial embolization is an effective therapy for these patients.

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Successful outcome after combined chemotherapeutic and surgical management in a case of esophageal cancer with breast and brain relapse

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Abstract

Esophageal cancer (EC) is a highly lethal disease. Approximately 50% of patients present with metastatic EC and most patients with localized EC will have local recurrence or develop metastases, despite potentially curative local therapy. The most common sites of distant recurrence are represented by lung, liver and bone while brain and breast metastases are rare. Usually patients with advanced disease are not treated aggressively and their median survival is six months. We report a woman patient who developed breast and brain metastases after curative surgery. We treated her with a highly aggressive chemotherapeutic and surgical combination resulting in a complete remission of the disease even after 11-year follow-up. We think that in super selected patients with more than one metastasis, when functional status is good and metastases are technically resectable, a surgical excision may be considered as a salvage option and chemotherapy should be delivered to allow a systemic control.

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Key words: Esophageal cancer; Breast and brain metastases; Combined chemotherapeutic and surgical treatment

Santeufemia DA, Piredda G, Fadda GM, Cossu Rocca P, Costantino S, Sanna G, Sarobba MG, Pinna MA, Putzu C, Farris A. Successful outcome after combined chemotherapeutic and surgical management in a case of

INTRODUCTION

Esophageal cancer (EC) is a highly lethal disease, with an estimated annual incidence of 14 550 new cases and 13 770 related deaths in 2006 in the USA^[1]. Approximately 50% of patients present with metastatic disease and most patients with localized EC will have local recurrence or develop metastases, despite potentially curative local therapy^[2].

The most common sites of distant recurrence are represented by lung, liver and bone^[3] while brain and breast metastases are rare. Brain metastases have been reported only in 3.6% of all resected patients^[4] but their incidence in all patients with EC is higher^[5].

Extra mammary malignancies rarely metastasize to the breast, usually in patients with disseminated neoplasms^[6] and according to literature data, only few cases of breast metastases from EC have been reported so far^[7-9].

The case presented here is a woman with EC who underwent surgery and later developed synchronous solitary breast and brain metastases. She showed uncommon sites of recurrence of the disease. Generally, patients with EC are not treated aggressively in the presence of advanced diseases. Nevertheless, we performed a highly combined aggressive chemotherapeutic and surgical approach resulting in a complete remission of the disease even after 11-year follow-up. Together with the clinical case description, considering the rarity of the event, we discussed the features connected with the diagnosis and management of such an uncommon presentation of the metastatic disease.

CASE REPORT

In June 1995 a 51-year-old white woman patient with EC was admitted to our hospital. She complained of severe dysphagia and dyspeptic disorders associated with a weight loss. Her history revealed recent onset of dysphagia,



Figure 1 Barium swallow shows the presence of a stenotic trait in the middle thoracic esophagus.

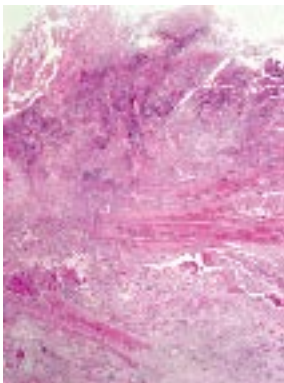


Figure 2 Esophageal histological sample (haematoxylin-eosin stain 20 x) shows the presence of squamous cell carcinoma with involvement of muscular tunic.

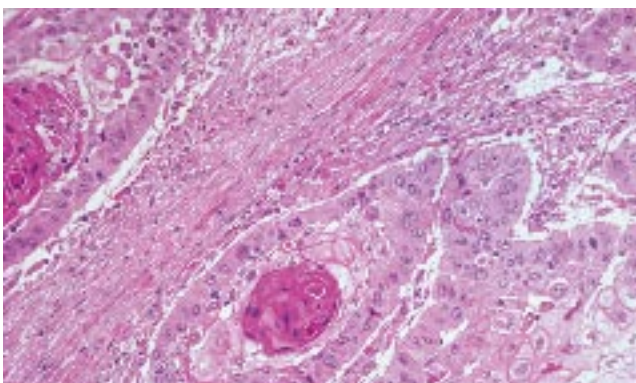


Figure 3 A squamous pearl at a higher magnification (haematoxylin-eosin stain 200 x).

present at first due to solid foods and then due to fluids as well. The difficult food intake caused a subsequent 5 kg weight loss during the five months prior to our observation. Since she had no other specific complaints, we decided to submit our patient to instrumental checks.

Barium swallow showed the presence of a 4 cm stenotic trait in the middle thoracic esophagus (Figure 1) and a next esophagogastroduodenoscopy revealed a vegetant mass in the esophageal lumen causing the stenosis. A biopsy of the mass was performed and histologic examination revealed a squamous esophageal carcinoma (Figures 2 and 3). The tumor marker values were increased and carbohydrate antigen 19-9 (CA19-9) was 250 U/mL (normal < 40 U/mL) while carcinoembryonic antigen (CEA) was 24

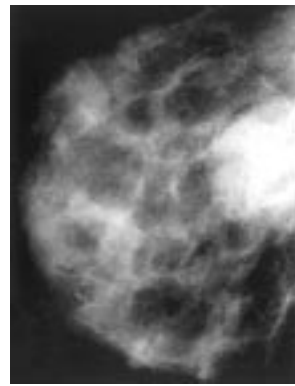


Figure 4 Mammography shows a well-defined nodule without calcification.

μg/L (normal < 3 μg/L). Other laboratory findings were normal.

Total body CT scan, abdominal ultrasonography and bone scintigraphy revealed that the disease was at stage IIa, we therefore decided to treat the patient surgically. In July 1995, subtotal esophagectomy was performed with dissection of lymph nodes and retrosternal reconstruction using tubulized gastric stump. Surgical operation was radical and the lymph nodes were negative for metastases. Histological examination confirmed the presence of squamous carcinoma and pathological stage of the disease was T₂ N₀ M₀. The woman underwent periodical clinical checks.

In November 1995, the patient noted the presence of an approximately 3 cm × 3 cm nodule in the upper lateral quadrant of the left breast. At clinical examination, it appeared as a hard mass not fixed to the surrounding structures. The tumor marker carbohydrate antigen 15-3 (CA15-3) was normal. A mammography confirmed the presence of the nodule (Figure 4) and fine-needle aspiration cytology under ultrasound guidance was performed, indicating the presence of squamous carcinoma cells.

Total body CT scan requested for restaging showed also a 1 cm × 1 cm focal lesion in the frontal right lobe of the brain. Therefore, we treated the woman with a palliative chemotherapy schedule based on cisplatin (25 mg/m² iv a day) from d 1 to d 5, and 5-fluorouracil (1000 mg/m² iv a day) from day 1 to day 5 by continuous infusion every four weeks^[10]. After the first cycle administration we observed remarkable reduction of the breast metastasis at clinical examination.

Taking into account either the palliative medical treatment or her relatively young age and good general conditions, we decided to perform surgical excision of both metastases followed by chemotherapy. In the following ten days, either tumorectomy or brain metastasectomy of the breast lesion was performed. Histological examination (Figures 5 and 6) revealed the presence of esophageal metastatic tumor. No surgical complication occurred. One month later, we resumed chemotherapy and further five courses of treatment were delivered.

In the following time the patient began to start again her daily activities and underwent periodical clinical and instrumental checks, which were always negative. The woman enjoyed good health after eleven years.

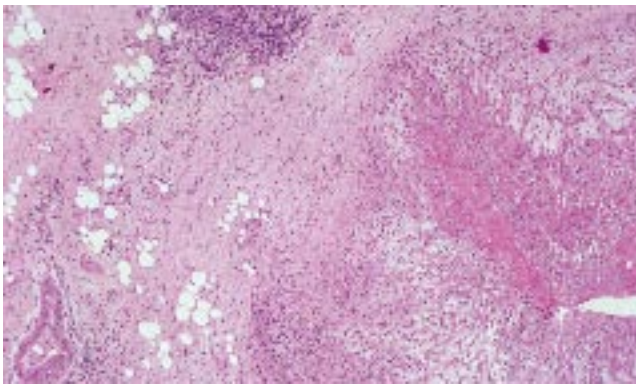


Figure 5 The right side of breast histological sample shows an infiltrating squamous carcinoma's focus with peritumoral inflammation while the left side shows a normal mammary duct section (haematoxylin-eosin stain 40 x).

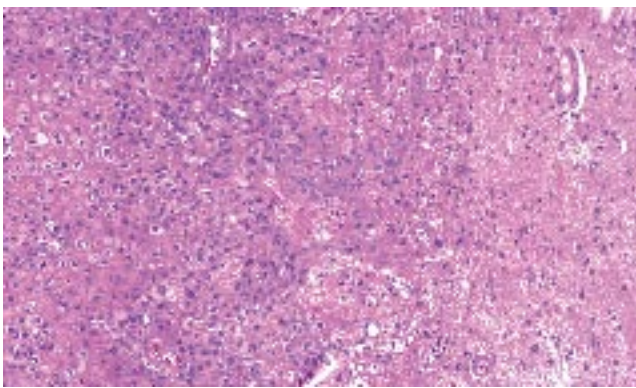


Figure 6 Squamous carcinoma infiltrating the cerebral parenchyma (haematoxylin-eosin stain 100 x).

DISCUSSION

EC is treatable but rarely curable. Patients with metastatic EC have a median survival time of six months^[11]. Moreover the reported 5-year survival rate ranges from 20% to 36% after intentionally curative surgery^[12] due to a high rate of either local or distant recurrence. Distant metastasis rate is reported to be 26% within 20 mo after radical surgery^[13]. Early metastatic relapse after complete resection of any apparently localized primary tumor indicates that micrometastatic tumor cell spread at the time of surgery is undetectable by current staging methods and routine histopathology^[14].

Commonly EC metastasizes to the lungs, liver and bone^[3] but rarely to the brain^[4] and exceptionally to the breast^[7-9]. We observed an unusual metastatic pattern with both solitary breast and brain single metastases in our patient. Breast metastases from non-mammary malignancies are very rare and their incidence ranges from 0.5% to 5.1% of all breast tumors^[7]. They usually occur in the upper outer quadrant^[6] and their prognosis is poor^[8,9].

Clinical and radiological aspects of secondary breast tumors are heterogeneous, though in most cases they appear as a solitary palpable, usually mobile mass. Mammographic finding consists of a round, dense and well-circumscribed mass, without spiculation, microcalcification or skin thickening. Furthermore, growth

is usually rapid^[15]. However, differential diagnosis is needed for correct treatment choice and it is reasonable to obtain a histological or cytological sample of the lesion.

The presence of metastatic lesions in any patient is an indication for a systemic treatment of primary cancer. Anyhow it has been reported that curative surgery for breast metastasis is a correct choice of treatment if metastases in other sites are controlled by other modalities^[7].

For the case reported here, we made a disease restaging which showed concomitant presence of a brain metastasis because of the detection of her breast metastasis. This feature represents a further worsening of the prognosis of our patient. As a matter of fact patients with brain metastases from EC have a median survival time of 3.9 mo^[16]. However it has been reported that an intensive brain tumor treatment may result in a longer survival time of selected patients with good Karnofsky performance status (KPS)^[17] after excision of a single brain metastasis from EC^[18]. Even the presence of multiple or recurrent brain metastases does not automatically contraindicate surgery because it can prolong survival and enhance the quality of life^[19]. Currently surgery followed by whole brain radiation therapy (WBRT) is considered the standard treatment^[20] for a single brain metastasis.

For the case reported here, we began chemotherapy in order to obtain a systemic control of the disease. Since we observed a remarkable reduction of the breast lesion after only one course of chemotherapy, we modified our treatment plan. Since the patient was relatively young and in good functional status, we decided to perform radical excision of her metastatic EC followed by chemotherapy. Brain radiotherapy was not performed. This combined aggressive chemotherapeutic and surgical approach resulted in a complete remission of the disease even after 11-year follow-up.

Generally, the prognosis of patients with recurrent EC is poor, but in some cases surgical resection, chemotherapy or radiotherapy has been proven effective^[21].

Our experience suggests that when functional status is good and metastases are technically resectable, surgical excision may be considered in selected patients with more than one metastasis and chemotherapy should be delivered to allow a systemic control.

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Collision tumor of the rectum: A case report of metastatic gastric adenocarcinoma plus primary rectal adenocarcinoma

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Abstract

Collision tumors are thought to arise from the accidental meeting and interpenetration of two independent tumors. We report here a highly unusual case of a 61-year old man who had a unique tumor that was composed of a metastatic adenocarcinoma from the stomach to the rectum, which harbored a collision tumor of primary rectal adenocarcinoma. The clonalities of the two histologically distinct lesions of the rectal mass were confirmed by immunohistochemical and molecular analysis. Although histologic examination is the cornerstone in pathology, immunohistochemical and molecular analysis can provide evidence regarding whether tumors originate from the same clone or different clones. To the best of our knowledge, this is the first reported case of such an occurrence.

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Key words: Collision tumor; Rectum; Clonality

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INTRODUCTION

The term collision tumor refers to two coexisting, but independent tumors^[1]. Malignant neoplasms originating from two or more distinct topographic organs may form a collision tumor. A possible explanation for this is field cancerization, which occurs due to long-term exposure to carcinogens, whereby multiple carcinogenic transformations give rise to genetically unrelated secondary primary tumors with independent mutations^[2,3] and thus, the chance of tumor collision may be increased. However, there is no explanation for the occurrence of many collision tumors. As most diagnoses are made based on the histology alone, the question is whether histologic classification can accurately reflect the molecular findings in these tumors. If two tumors arise independently and are associated with coincidence only, the genetic alterations are expected to be different from each other because of the different tumor origins. We report here a rare collision tumor of the rectum that was composed of a rectal adenocarcinoma within the metastatic gastric adenocarcinoma. Furthermore, in an effort to find the molecular evidence both for the histologic diagnosis of this collision tumor and for the clonality of the two separate components, we characterized the molecular alterations of each tumor component by examining the microsatellite instability (MSI) and loss of heterozygosity (LOH), and performed an immunohistochemical analysis as well. The findings of this tumor represent an entity that has never been described at this location.

CASE REPORT

A 61-year old man presented with postprandial epigastric pain for 5 mo. Upper gastrointestinal endoscopy suggested an ulcerofungating tumor spreading from the gastric body and antrum to near the esophagogastric junction, and biopsy revealed a poorly differentiated adenocarcinoma. The patient underwent radical total gastrectomy for the gastric cancer with regional lymph node dissection. During the operation, the rectum showed a tumor that was considered to be a distant metastasis from the gastric cancer. So, low anterior resection of the rectum with regional lymph node dissection was also performed for the distant metastasis of the gastric carcinoma.

The resected rectum revealed a relatively ill-defined ulcerofungating mass measuring 7 cm × 6 cm. Sectioning

Table 1 Results of immunohistochemistry for primary rectal carcinoma component and both primary and metastatic carcinoma components of the stomach

Immunohisto-chemical markers	Antibody			Results		
				Rectal tumor		Primary stomach
	Source	Clone	Dilution	Primary rectum	Metastatic stomach	
CK7	DakoCytomation	OV-TL	1:200	Positive	Negative	Negative
CK20	DakoCytomation	Ks20.8	1:50	Positive	Positive	Positive
p53	DakoCytomation	DO-7	1:50	Positive	Positive	Positive
MUC2	Novocastra	Ccp58	1:500	Negative	Positive	Positive
MUC5AC	Novocastra	CLH2	1:500	Negative	Negative	Negative
CDX2	Novocastra	CDX2-88	1:100	Positive	Positive	Positive

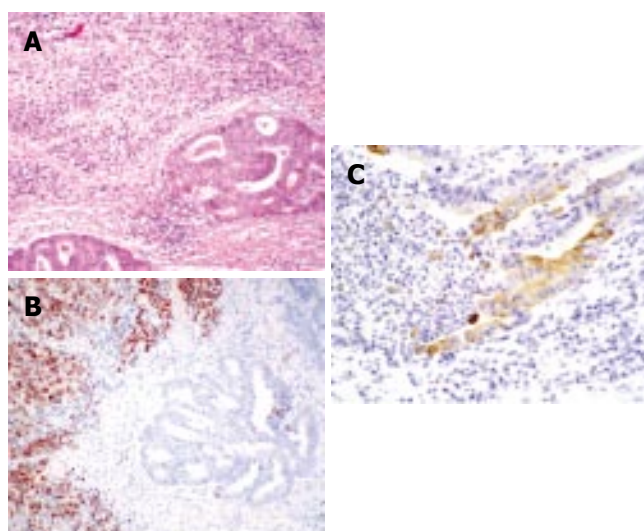


Figure 1 Histological features of rectal tumor showing the interface of a tubular adenocarcinoma component of primary rectal cancer (right) and a poorly-differentiated adenocarcinoma component metastasized from the stomach (left) (hematoxylin and eosin; $\times 40$) (A), immunohistochemical analysis showing positive MUC2 in metastatic gastric carcinoma and negative MUC2 in primary rectal carcinoma ($\times 200$) (B), and negative CK7 in metastatic gastric carcinoma and positive CK7 in primary rectal carcinoma ($\times 200$) (C).

revealed a whitish granular infiltrating tumor with extension into the perirectal soft tissue. Any regional differences of the tumor were not grossly identified. The resected stomach revealed a Borrmann type IV mass, measuring 14 cm \times 14 cm, at nearly the entire gastric wall. Microscopically, the rectum showed a larger component of poorly differentiated adenocarcinoma with a focal signet-ring cell appearance involving the entire rectal wall. A smaller component of well-differentiated tubular adenocarcinoma invading into the perirectal soft tissue was noted within the poorly differentiated adenocarcinoma. Both components collided with each other with no intermingling at their interface (Figure 1A). The surgical margins were tumor-free. Multiple regional lymph nodes ($n = 28$) showed metastatic adenocarcinoma ($n = 26$), of which two revealed feature of well-differentiated tubular adenocarcinoma and 24 revealed poorly-differentiated adenocarcinoma. The stomach mass was an invasive, poorly-differentiated adenocarcinoma invading into the perigastric soft tissue and showing the same histologic features as the larger component of the rectal tumor. The

well-differentiated tubular adenocarcinoma cells seen in the rectal wall were not found in the gastric wall. The proximal resection margin was tumor-involved, but the distal resection margin was tumor-free. Multiple regional lymph nodes ($n = 60$) showed poorly-differentiated metastatic adenocarcinoma ($n = 47$).

Immunohistochemical staining was performed to distinguish the two components of the rectal tumor. The characteristics of the antibodies used in this study and the results are presented in Table 1 as well as in Figure 1B and C. In summary, the primary gastric carcinoma as well as the metastatic gastric carcinoma in the rectum displayed both strong and diffuse staining for MUC2, but negative staining for cytokeratin 7 (CK7), whereas the primary rectal carcinoma component showed focal positive immunoreactivity for CK7, but negative staining for MUC2. The distribution of immunostaining was well correlated with the histologic distinction between metastatic gastric and primary rectal carcinoma components in the collision tumor.

The tissue of both tumors and their non-tumor counterparts were scraped from 10 μ m-thick formalin-fixed, paraffin-embedded sections, and then genomic DNA was extracted using the DNeasy tissue kit (Qiagen, Hilden, Germany). DNA sample pairs were amplified using the microsatellite instability MSI/LOH starter kit (Applied Biosystems, Foster City, CA, USA). Genetic stability was analyzed using the Bethesda reference panel that includes BAT25, BAT26, D2S123, D5S346 and D17S250^[4]. Both the primary and metastatic gastric carcinoma components as well as the primary rectal carcinoma component showed microsatellite stability (Figure 2). LOH analysis was carried out using 3 polymorphic microsatellite repeat markers including D2S123, D5S346 and D17S250. A value below 0.6 or above 1.6 was interpreted as evidence of LOH, whereas values between these figures were considered retention of heterozygosity. LOH was found in both primary and metastatic gastric carcinoma components, but not in primary rectal carcinoma component (Table 2). Eight months after surgery, the patient died of recurrent gastric cancer.

DISCUSSION

Collision tumor is considered as a double tumor showing a 'side by side' or 'one upon another' pattern. It can

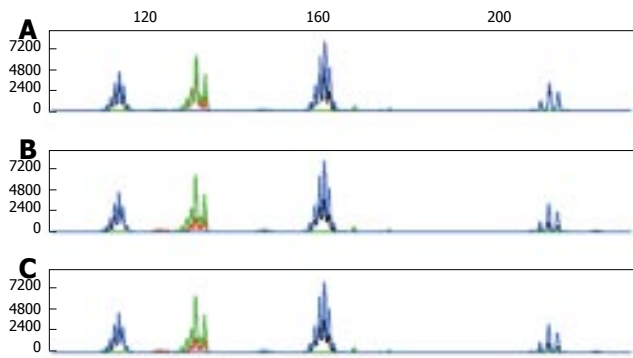


Figure 2 Microsatellite instability phenotype analysis showing no microsatellite instability in the primary rectal tumor (A), the metastatic gastric tumor (B) and the primary gastric tumor (C). Blue and green line: Normal tissue; Black and red line: Tumor tissue.

Table 2 Results of loss of heterozygosity analysis for primary rectal carcinoma component and both primary and metastatic carcinoma components of the stomach

Microsatellite marker	Chromosomal region	Tumor suppressor gene	Loss of heterozygosity		
			Rectal tumor		Primary stomach
			Primary rectum	Metastatic stomach	
D2S123	2p16	hMSH2	No (0.95)	No (0.93)	No (1.03)
D5S346	5q21	APC	No (1.31)	Yes (0.50)	Yes (0.54)
D17S250	17q11.2-12	P53	No (1.01)	Yes (0.41)	Yes (0.43)

occur within the same organ, or in adjacent organs, or in conjunction with systemic malignancy^[5]. Several hypotheses have been suggested as the mechanisms for collision tumor. The simplest is that two primary tumors occur in continuity by an accidental “meeting”. Two different tumors may also contiguously develop because the region is altered by the same carcinogenic stimuli. Another hypothesis is that the presence of the first tumor alters the microenvironment, making the development of the second adjacent tumor more likely^[5]. In our case, because the primary rectal cancer occupied a smaller portion of the lesion within the larger portion of metastatic gastric cancer, suggesting that metastatic gastric carcinoma to the rectum probably makes some changes in microenvironment and metabolic condition of the rectum, in which a second primary rectal cancer may have developed in association with a previous metastatic focus rather than a metastasis harboring rectal adenocarcinoma. Therefore, we can postulate that substances produced by gastric adenocarcinoma stimulate the immediate adjacent mucosa to undergo increased proliferation and neoplastic transformation.

Collision tumor needs to be distinguished from composite tumor, which is characterized by two divergent lineages originating from the same neoplastic clonal proliferation^[1], because different treatments are warranted depending on the type of collision tumor encountered^[6]. The behavior of collision tumor depends on the individual elements. Definitive conclusion could not be drawn with regard to the prognosis of this type of collision tumor

in our case. However, it is important to differentiate between a case with rectal carcinoma coincidentally having a metastatic gastric carcinoma component and a case with only a primary rectal carcinoma component because of the difference in prognosis. The prognosis of patients with only primary rectal carcinoma is determined by the staging at diagnosis and it is likely to be more favorable than that of patients with an additional metastatic gastric adenocarcinoma in the rectum.

Genetic analysis provides evidence regarding whether the tumors originate from the same clone or from different clones. The panels for each anatomically different site of tumor are then compared to identify the conserved and unique mutations. If the mutational profiles are predominantly unmatched, the diagnosis of a second primary tumor can be established. In an effort to determine the clonality of the two separate components in this patient's rectal tumor, we characterized the molecular alterations of each tumor component, by MSI and LOH analysis. It has been recently reported that the pattern of MSI findings is a useful tool in determining whether a patient has double primary tumors or a single clonal tumor with metastasis^[7]. Kim *et al*^[8] reported that high coincident MSI is observed in 17.7% of patients with colon and stomach cancers, suggesting that a genetic defect of mismatch repair deficiency may be responsible for a small subset of double primary cancers of the colorectum and stomach. In this case, we could not find any discriminating pattern of MSI in both primary and metastatic gastric carcinoma components, as well as in primary rectal carcinoma component. The clonal evolution of cancer can be followed up by using markers that identify LOH. However, carcinogenesis is most often not a single event, but rather the result of many mutations that accumulate over time. Blaker *et al*^[9] reported that LOH patterns of primary colon cancer and metastatic tumors are different in about half of the cases. In our case, LOH analysis showed that the two components of rectal tumor were collision tumor.

The mucin gene family consists of at least nine MUC genes whose tissue distribution has mainly been studied with antibodies that recognize the core protein of different mucins. At immunohistochemical level, the expressed main mucin types are MUC1 for the intestinal type, MUC5AC for the diffuse type, and MUC2 for the mucinous type in gastric cancer^[10]. MUC2 expression has been shown to be significantly lower in non-mucinous colorectal cancer^[11]. Different expressions of various types of CK in tumors at different primary sites can be a clue to the origin of a neoplasm. It has been reported that carcinomas of the colon generally express CK20, whereas the CK7 expression is usually negative, and the expressions of CK7 and CK20 in carcinomas of the stomach have yielded more variable results^[12]. However, carcinomas of a gastrointestinal origin exhibit overlapping and heterogeneous expressions of each mucin and also CK, and there is no definitively consistent immunoreactivity pattern. In this study, primary gastric carcinoma as well as metastatic gastric carcinoma in the rectum displayed strong and diffuse staining for MUC2, but negative staining for CK7, whereas the primary rectal carcinoma component showed focal

positive immunoreactivity for CK7, but negative staining for MUC2. The distribution of immunostaining was significantly correlated with the histologic distinction between metastatic gastric and primary rectal carcinoma components in collision tumor.

To the best of our knowledge, although one of the tumors with a significantly elevated risk is colorectal cancer after the diagnosis of stomach cancer, rectal collision tumor with primary rectal adenocarcinoma and metastatic gastric adenocarcinoma is an entity that has not been previously described at this location. Our immunohistochemical and molecular approach clearly demonstrates that the two components of adenocarcinoma of the rectum have a different clonality.

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Mucosa-associated lymphoid tissue lymphoma of the transverse colon: A case report

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Abstract

We herein present a case of a 75-year-old female with mucosa-associated lymphoid tissue (MALT) lymphoma of the transverse colon with the stage IE (Ann Arbor classification). Colonoscopy revealed the tumor's appearance as a IIa plus IIc-like early colon cancer as defined according to the macroscopic classification of the Japanese Research Society for Cancer of Colon, Rectum and Anus, measuring less than 2 cm in diameter. Histologically, the tumor was diagnosed as MALT lymphoma because of the presence of lymphoepithelial lesions consisting of diffuse proliferation of atypical lymphocytes and glandular destruction. The majority of these lymphocytes immunohistochemically stained for the B-lymphocyte marker. The patient first underwent *H. pylori* eradication therapy with Lansap®. However, the tumor size gradually increased over the next 4 mo and the patient eventually underwent surgical resection. The operative procedure included a partial colectomy with dissection of the paracolic lymph nodes. The tumor measured 45 mm x 30 mm in diameter and histological examination showed that the lymphoma cells had infiltrated the muscle layer of the colon without nodal involvement. The patient has had no recurrence postoperatively without any chemotherapy.

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Key words: Mucosa-associated lymphoid tissue; Malignant lymphoma

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INTRODUCTION

The term mucosa-associated lymphoid tissue (MALT) lymphoma was first introduced by Isaacson and Wright^[1] in 1983. This entity includes low-grade gastric B-cell lymphoma and immunoproliferative small intestinal disease. MALT lymphomas occur in a variety of extra-nodal organs, such as the gastrointestinal (GI) tract and the non-GI tract, in which the stomach is the most common site^[2,3]. Since convincing evidence has been presented showing the relationship between *H. pylori* and gastric MALT lymphoma, the therapeutic strategy has been altered for patients with gastric MALT lymphoma in the early stages^[4-8]. In contrast, a treatment for colonic MALT lymphoma has not yet been established. In the present report, we describe a case of colonic MALT lymphoma which did not respond to *H. pylori* eradication treatment and, therefore, underwent a surgical resection, and also provide a literature review on this rare entity.

CASE REPORT

A 75-year-old female was admitted to Nagasaki Prefectural Shimabara Hospital for surgical treatment of MALT lymphoma of the transverse colon on June 21, 2005. The patient had shown a positive fecal occult blood test on January 20, 2005 without any clinical symptoms and signs. Her past histories included hypertension, diabetes mellitus, and cholecystolithiasis. The results of complete blood counts, blood chemistries and tumor markers, such as carcinoembryonic antigen, were all within the normal limits. Colonoscopy revealed the tumor's appearance as IIa plus IIc-like early colon cancer^[9], measuring less than 2 cm in diameter, in the transverse colon (Figure 1). Biopsy specimens histologically showed lymphoepithelial lesions with diffuse proliferation of atypical small lymphocytes and some glandular destruction. These lymphocytes immunohistochemically showed diffusely positive staining for L-26 (Figure 2) and bcl-2, but negative staining for CD3, CD5, CD10, CD79a, UCHL-1 and cyclin D1. These findings were compatible with MALT lymphoma of the colon. Barium enema showed a flat-elevated lesion in the transverse colon (Figure 3). Abdominal and chest CT demonstrated neither abnormal lesions nor

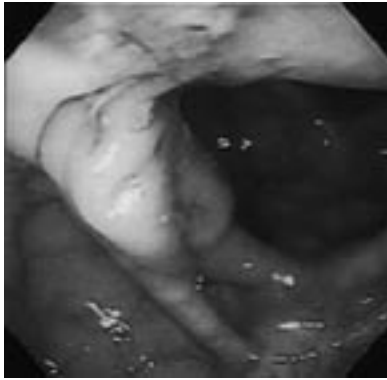


Figure 1 Colonofiberscopy showing the tumor's appearance as a IIa plus IIc-like early colon cancer.



Figure 3 Barium enema showing a flat and well-circumscribed tumor in the transverse colon (arrows).

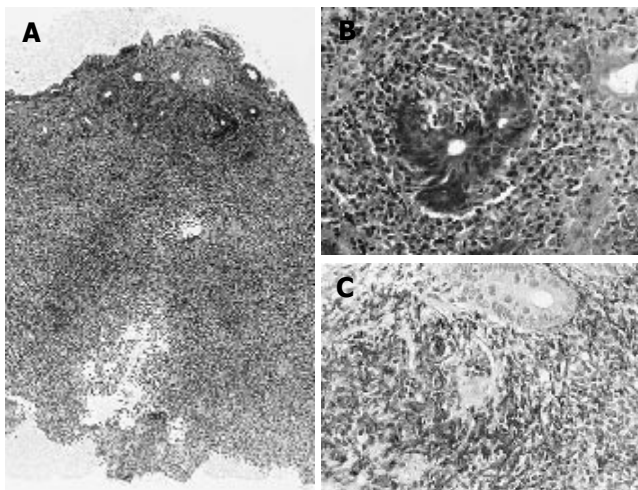


Figure 2 Biopsy specimens histologically showing diffuse proliferation of atypical small lymphocytes in the mucosal layer (A: x 40 magnification, HE) and glandular destruction (B: x 200 magnification, HE). These lymphocytes immunohistochemically showing diffusely positive staining for L-26 (C: x 200 magnification, ABC method).

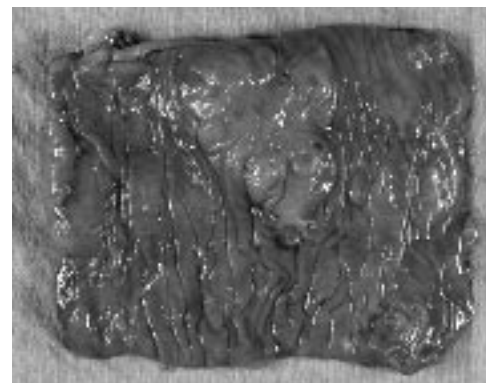


Figure 4 Resected specimens showing a flat-elevated tumor with slight depression, measuring 45 mm x 30 mm in diameter.

enlargement of lymph node. According to the Ann Arbor classification, this MALT lymphoma belonged to the stage IE. The patient first underwent *H. pylori* eradication therapy with Lansap[®] because of positive reaction to a urea breath test (UBT). However, over the course of 4 mo, the tumor gradually increased in size, although *H. pylori* were eradicated. The patient was considered to be a non-responder to eradication therapy, and was indicated for surgical resection. A partial colectomy with dissection of the paracolic lymph nodes was performed on June 23, 2005 (Figure 4). The tumor grossly appeared to be a IIa plus IIc-like early colon cancer, measuring 45 mm × 30 mm in diameter. Resected specimens were histologically and immunohistochemically reconfirmed to be MALT lymphoma without nodal involvement. The lymphoma cells infiltrated mainly into the mucosa and submucosal layer, and partly infiltrated into the muscular layer of the colon (Figure 5). The patient has had no recurrence postoperatively without any chemotherapy.

DISCUSSION

MALT lymphoma is defined as extra-nodal marginal zone B-cell lymphoma of MALT type in peripheral



Figure 5 The lymphoma cells mainly infiltrated into the mucosal and submucosal layers, and partly infiltrated into the muscular layer of the colon (x 20 magnification, HE).

B-cell lymphoma according to morphologic features, immunophenotype, genetic features, postulated normal counterpart, and clinical features^[10,11].

H. pylori eradication therapy is currently widely recognized as an initial therapy in cases with low-grade (stage I) gastric MALT lymphoma^[12-14]. In contrast, it has not yet been clarified whether *H. pylori* eradication, or chemotherapy, or surgery should be performed in colonic MALT lymphoma compared with gastric MALT lymphoma, because the colorectal MALT lymphomas are rare. The individual clinical details of colorectal MALT lymphoma are summarized in Table 1^[15-27]. Some reports have

Table 1 Colorectal MALT lymphoma in the English literatures

Author	Yr	Age/Sex	Location/Size	Symptoms/Signs	<i>H pylori</i>	Treatment	Outcome
Schmid ^[15]	1994	65/M	S/2.5 cm	ND	ND	Polypectomy	NED for 9 mo
	1994	47/M	T/1.5 cm	ND	ND	Polypectomy and chemotherapy	NED for 24 mo
	1994	64/M	T/1.5 cm	ND	ND	Left hemicolectomy	Died, 7 d (Cardiac failure)
Matsumoto ^[16]	1997	72/F	R/ND	Rectal bleeding	+	Eradication	NED for 12 wk
Yasui ^[17]	1999	76/M	C/30 × 15 mm	Fecal occult blood	ND	Partial resection ²	ND
Orita ^[18]	1999	64/F	R/3.4 × 4.8 cm	Fecal occult blood	ND	Abdominoperineal resection	NED for 35 mo
Inoue ^[19]	1999	62/F	R/ND	Hematochezia	-	Eradication	NED for 53 wk
Raderer ^[20]	2000	67/M	D ¹ /1.5 cm	None	+	Eradication	NED for 4 mo
Hasegawa ^[21]	2000	72/F	S/3.0 × 1.5 cm	Abdominal pain, fever	ND	Sigmoidectomy	ND
Yoshimura ^[22]	2002	74/M	T/8 × 4 cm	Fecal occult blood	ND	Chemotherapy	NED for 20 mo
Nakase ^[23]	2002	66/F	D, S, R/ND	Hematochezia	-	Eradication	NED for 1.5 yr
	2002	33/F	R/ND	Fever, hematochezia	-	Eradication	NED for 10 mo
	2002	62/F	R/ND	Hematochezia	-	Eradication	NED for 6 mo
Hisabe ^[24]	2002	70/F	R/1.5 cm	Abdominal discomfort	-	Eradication	NED for 20 mo
Takada ^[25]	2003	44/M	C/1.1 × 0.9 cm	Fecal occult blood	-	Partial resection ²	ND
Lee ^[26]	2005	47/M	R/ND	Tenesmus, mucoid stool	-	Chemotherapy and radiation	NED for 3 mo
Kikuchi ^[27]	2005	71/M	R/3.5 cm	Fecal occult blood	-	Eradication ³	NED for 12 mo
	2005	80/F	C, R/2.5 cm	Anal bleeding	-	Eradication	NED for 6 mo
	2005	70/F	R/1.5 cm	Abdominal discomfort	-	Eradication	NED for 20 mo
Our case	2006	75/F	T/4.5 × 3.5 cm	Fecal occult blood	+	Partial resection after eradication	NED for 12 mo

Location; R: Rectum, S: Sigmoid colon; D: Descending colon; T: Transverse colon; C: Cecum; ND: Not described; NED: No evidence of disease. ¹This patient had MALT lymphomas in the stomach and the descending colon, simultaneously. ²These patients performed laparoscopy-assisted colon resection. ³This patient has received repeated eradication.

described the successful regression of colorectal MALT lymphoma by means of eradication therapy in *H pylori*-positive patients^[16,20]. Even in cases with colorectal MALT lymphoma negative for *H pylori*, the regression of the tumor was also recognized as a result of *H pylori* eradication therapy^[19,23,24,27]. In prospective studies of gastric MALT lymphoma, eradication therapy was not effective in patients negative for *H pylori*^[28,29]. Grunberger *et al*^[30] reported that antibiotic eradication therapy was not effective in patients infected with *H pylori* suffering from extra-gastric MALT lymphoma. Therefore, they suggested that *H pylori* did not play a role in the development of extra-gastric MALT lymphomas. Similarly, antibiotic eradication therapy was not effective in our present case. These results may suggest that colorectal MALT lymphomas are not directly related to *H pylori* infection, while gastric MALT lymphomas are strongly associated with *H pylori* infection. In the future, a definite pathogenesis of colorectal MALT lymphoma should be clarified when cases of colorectal MALT lymphomas have accumulated. As a speculation, colorectal MALT lymphomas may be caused by unknown antibiotic-sensitive microorganisms other than *H pylori*, although that is not clear. In gastric MALT lymphoma, indeed, *Helicobacter heilmannii*-associated MALT lymphoma other than *H pylori* has been reported to be completely regressed by eradication therapy^[31].

Surgical resection is mandatory when a colorectal MALT lymphoma does not respond to eradication therapy or chemotherapy, and it is localized without dissemination.

In conclusion, the present case with colonic MALT lymphoma eventually underwent surgical resection of the colon after the failure of eradication therapy. Surgical intervention is now the procedure of choice for colorectal MALT lymphoma because its pathogenesis and therapeutic strategy have not yet been established.

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Unusual site of recurrent musculoskeletal hydatid cyst: Case report and brief review of the literature

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Abstract

A case of a large multiplex recurrent hydatid cyst involving the left gluteal muscle and the left iliopsoas, accompanied with degeneration of the musculature of the left upper leg is presented along with a review of the relevant literature. Very few such cases have been reported worldwide. The presented case is also distinguished by the involvement of muscles of distant anatomic areas.

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Key words: Echinococcosis; Hydatid disease; Musculoskeletal hydatid

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INTRODUCTION

Echinococcosis is a zoonotic infection caused by tapeworms of the genus *Echinococcus* which inhabits in the small intestine of carnivores. The adult worms produce eggs that are released with the feces and spread in various ways, such as through the wind, water or flies^[2]. After ingestion by the host, the embryos migrate through the intestinal wall and are either arrested in the capillary bed of the liver developing into liver cysts, or manage to penetrate into systemic circulation thus ending up in remote organs. The lung, the brain, and the muscles or

bones are the more frequently involved distant organs. Due to their physiologic role as capillary filters and their vast capillary volume, the liver and lung are most often affected. Other manifestations are found in 15% of the patients, with the skeletal system making up for 1%-4% of all cases^[7]. Voluntary muscles are a very rare site of infection, counting for less than 1% of total^[5].

In this report, we present a case of a large multiplex recurrent hydatid cyst involving the left gluteal muscle and the left iliopsoas, accompanied with degeneration of the musculature of the left upper leg. To our knowledge, less than 50 such cases have been reported in the literature worldwide. The presented case is also distinguished by the involvement of muscles of distant anatomic areas, while no liver, lung or bone lesions were identified.

CASE REPORT

A 78-year-old Caucasian male was admitted to our clinic, with a swelling of the waist and the left upper leg. The patient had been operated four times over the past 18 years for echinococcosis of the musculature of the left upper leg. During this period, he suffered recurrent swellings of the thigh that were treated as abscesses. His past medical record was otherwise unremarkable.

On physical examination, a mass was located in the left waist and the upper part of the left gluteus muscle. A communicating fistula with a daughter cyst was identified in the median part of the left gluteus muscle. Another communicating fistula extended from the daughter cyst to 5 cm above the knee joint where a third cyst was palpable. The aforementioned findings were also visible on ultrasound (US) and MRI studies. The lesions of the primary and daughter cyst in the waist and the communicating fistula were located superficially at the fascia of the left gluteus muscle (maximum diameter 10 cm).

There were no signs of calcification in any of these cysts. No liver or lung manifestations were present. Anti-echinococcal IgG was positive for infection (title 2,2, positive if > 1.1, ELISA).

The patient was submitted to perform a complete cystopericystectomy of both primary and daughter cysts and radical excision of the fistula. The overlying skin was excised and the surgical wound was primarily closed. The lesions were easily dissected from the underlying fascia that did not need removal. The cysts contained purulent material. The wound was drained for 24 h. The surgical



Figure 1 Preoperative appearance of the lesion, where primary cyst, communicating fistulas and daughter cysts are discernible.

specimen was submitted for histopathological examination which confirmed the diagnosis. The postoperative course was uneventful and the patient was discharged on the 6th postoperative day. He has been followed up for 16 mo and no recurrent cyst was evident on CT and MRI scan.

DISCUSSION

Soft tissue hydatid disease is rare even in endemic areas, such as the Mediterranean. Intramuscular lesions in the absence of liver, lung or bone manifestations are most uncommon^[1,4]. In our case, we encountered an extended soft tissue disease with no signs of systemic infection but a history of multiple recurrences. To our knowledge, very few similar cases have been reported in the literature worldwide (Table 1). Recurrences had developed in the form of abscess-like lesions in multiple sites of the leg which is also uncommon in intramuscular infections. Quality of life of this patient was poor due to constant symptomatic hydatidosis, possibly a result of chronic local inflammation. He also experienced mobilization difficulties (Figure 1).

Determining the ideal therapeutic approach for a recurrent musculoskeletal hydatid cyst can be quite challenging for the general surgeon. Moreover, the rarity of the disease renders the decision making on the favorable treatment quite difficult. Conservative treatment, complete excision and simple drainage have all been suggested as adequate^[10]. Hydatid disease progresses slowly and is rarely life-threatening, especially when located in the soft tissue or muscles, thus supporting a more conservative therapeutic approach. Additionally, co-morbid conditions and advanced age of the patient as well as the surgeon's experience are of great importance for the final decision. However, in case the disease causes profound disabilities or mobilization problems, complete cystopericystectomy should always be considered.

The nature of the lesion should be well documented and evaluated. Radical surgical therapy is especially indicated in cases of unilocular manifestations as only this method offers hope of permanent cure^[9]. Therapeutic dilemmas could arise in cases of extended disease with many muscles or muscle layers in different sites of the body which are communicating *via* fistulas. Communication between lesions should always be suspected and revealed, even if primary and daughter cysts are distant. Complete surgical treatment should include the primary lesion, the daughter cysts and the communicating fistulas as a whole specimen.

Hydatid disease may occur anywhere in the musculo-

Table 1 Reported sites of intramuscular infection and bone, liver or lung involvement^[2,3]

Author	n	Site of infection	Liver/lung/bone involvement
Merkle <i>et al</i> ^[2]	8	Iliopsoas, left adductor musculature, left femur, left medial gluteal muscle, musculature of right upper leg	Yes
Rieber <i>et al</i> ^[8]	1	Paravertebral structures	Yes
Sennaroglu <i>et al</i>	1	Infratemporal	Yes
Von Sinner ^[11]	1	Pelvic	Yes
Torricelli <i>et al</i> ^[12]	14	Bone infection with adjacent soft tissue involvement in 12 cases	
Aydin <i>et al</i> ^[13]	1	Cerebral	Yes
Duncan <i>et al</i> ^[4]	1	Biceps brachii	No
Dahniya <i>et al</i> ^[3]	7	5 bone infections without soft tissue involvement, 2 primary intramuscular (left shoulder, rectus femoris and vastus lateralis)	No

skeletal system, from the big toe to the crown of the head^[5,6,8]. In endemic areas, echinococcosis should be always suspected and bared in mind in the differential diagnosis of cystic lesions in soft tissue, even if the radiological appearance is not typical. Once the diagnosis is established, the surgeon should consider performing a radical procedure aiming in minimizing the possibility of a recurrence.

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An alternative surgical approach to a difficult case of Mirizzi syndrome: A case report and review of the literature

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Abstract

Mirizzi syndrome (MS) is an uncommon complication of gallstone disease and occurs in approximately 1% of all patients suffering from cholelithiasis. The syndrome is characterized by extrinsic compression of the common hepatic duct frequently resulting in clinical presentation of intermittent or constant jaundice. Most cases are not identified preoperatively. Surgery is the indicated treatment for patients with MS. We report here a 71-year-old male patient referred to the surgical outpatient department for diffuse upper abdominal pain and mild jaundice (bilirubin rate: 4.2 mg/dL). Ultrasound examination revealed a stone in the cystic duct compressing the common hepatic duct. The patient had a history of gastrectomy for gastric ulcer 30 years ago. MRCP revealed a stone impacted in the cystic duct causing obstruction of the common hepatic duct by extrinsic compression. With these findings the preoperative diagnosis was indicative of MS. At laparotomy a moderately shrunken gallbladder was found embedded in adhesions containing a large stone which was palpable in the common bile duct. The anterior wall of the body of the gallbladder was opened by an incision which extended longitudinally along the gallbladder towards the common bile duct. The stone measuring 3.0 cm in diameter, was then removed setting astride a large communication with the common bile duct. A Roux-en-Y cholecysto-cholecho-jejunostomy was performed. The subhepatic region was drained. The patient had an uneventful recovery. He was discharged eleven days after operation and remained well after a 30-mo follow-up.

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Key words: Benign jaundice; Hepatic duct obstruction; Impacted gallstone; Cholecystobiliary fistula

INTRODUCTION

Mirizzi syndrome (MS) is a rare complication of long-standing cholelithiasis, which results from impaction of a large calculus or multiple small stones in the cystic duct or in the neck of the gallbladder causing extrinsic narrowing of the common hepatic duct. This condition may result in the clinical presentation of intermittent or constant jaundice. MS occurs in approximately 1% of all patients with cholelithiasis. Although modern imaging techniques are available, the majority of cases are identified during surgery. During the last two decades, 27 patients suffering from MS have been treated in our department^[1,2], we present here a case of a 71-year-old male patient with MS and a literature review.

CASE REPORT

A 71-year-old male patient was referred to the surgical outpatient department for diffuse upper abdominal pain and mild jaundice (bilirubin rate: 4.2 mg/dL). Ultrasound examination revealed a stone in the cystic duct compressing the common hepatic duct (Figure 1). The patient had a history of gastrectomy for gastric ulcer 30 years ago, thus ERCP was not feasible. MRCP revealed a stone impacted in the cystic duct causing obstruction of the common hepatic duct by extrinsic compression. With these findings the preoperative diagnosis was indicative of MS. At laparotomy a moderately shrunken gallbladder was found embedded in adhesions containing a large stone which was palpable in the common bile duct (Figure 2A). It was obvious that the local operative circumstances required great surgical care. Therefore the anterior wall of the body of the gallbladder was opened by an incision which extended longitudinally along the gallbladder towards the common bile duct. The cystic duct could not be identified. The stone measuring 3.0 cm in diameter was then removed setting astride a large

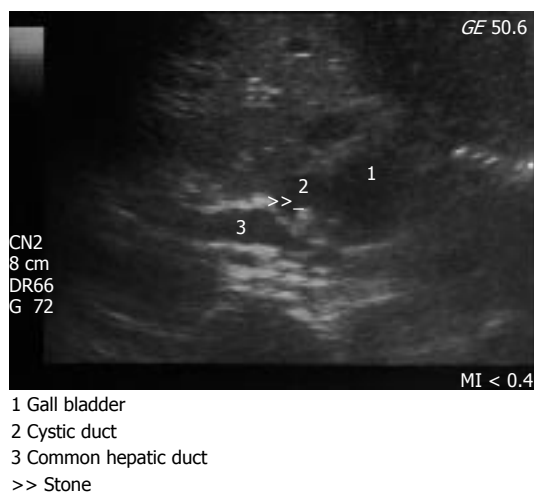


Figure 1 Ultrasound showing a stone compressing the common hepatic duct.

communication with the common bile duct (Figure 2B). Based on this finding and because the risk of stricture at the site of fistulae was significant, we decided to bypass the cholecystocholedochal fistula defect rather than to close it directly or by using a gallbladder flap for closing the opening of the common bile duct around a T-tube. A Roux-en-Y cholecysto-choledocho-jejunostomy was performed (Figure 2C). The subhepatic region was drained. The patient had an uneventful recovery. He was discharged on the 11th postoperative day and remained well after a 30-mo follow-up.

DISCUSSION

MS was first described in 1948 as obstructive jaundice due to a gallstone impacted in the cystic duct or Hartmann's pouch compressing the common hepatic duct^[3].

McSherry *et al*^[4] in 1982 suggested a subclassification of MS into two types. The first type concerns the external compression of the common hepatic duct by a calculus in the cystic duct or Hartmann's pouch, whereas in the second type the stone has entered partly or completely into the common bile duct, resulting in a cholecystocholedochal fistula. Furthermore, in 1989 a new classification of patients with MS and cholecystobiliary fistulae was presented by Csendes *et al*^[5], which includes four types: type I lesion includes those with external compression of the common bile duct; type II lesion is a cholecystobiliary fistula present with erosion of less than one third of the circumference of the bile duct; type III lesion is a fistula involving up to two-thirds of the duct circumference; type IV lesion is a complete destruction of the bile duct.

MS and cholecystobiliary fistulae therefore appear to be different, evolving stages of the same pathological condition, thus it is reasonable that Lubbers^[6] proposes that the term MS can now be abandoned, since it is only the first stage of a more complex process.

Gallstone erosion into the common duct is nevertheless a rare complication of cholelithiasis with an incidence rate ranging from 0.7% to 1.4% of all patients undergoing

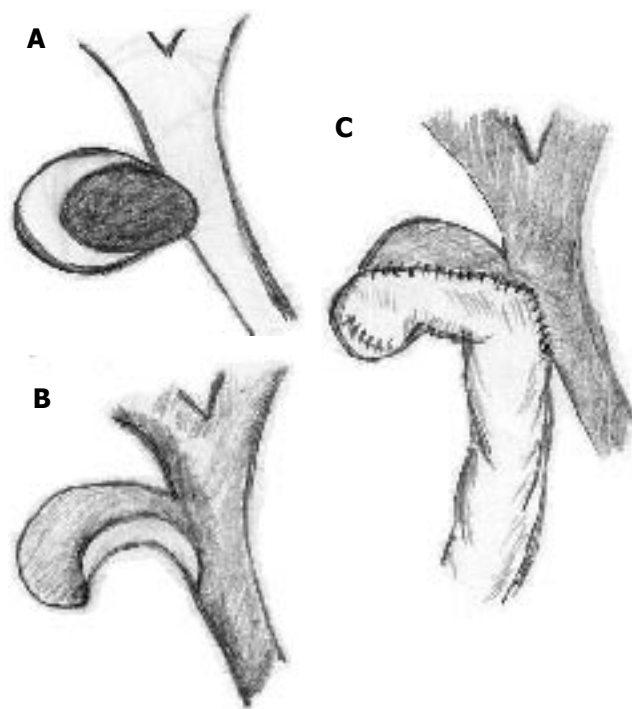


Figure 2 Schematic representation of the described technique during laparotomy (A, B) and Roux-en-Y cholecysto-choledocho-jejunostomy (C).

cholecystectomy^[7].

The clinical diagnosis of MS is difficult, since there are no pathognomonic patterns of presentation. Ultrasound is diagnostically the best screening method, with ERCP and/or MRCP to confirm the diagnosis. MRCP can be as good as ERCP in the diagnosis and its ability to delineate details of biliary structures, but its disadvantage compared to ERCP is its inability to confirm the presence of fistulae and does not afford therapeutic stenting. On the other hand, T₂ weighted sections can differentiate a neoplastic mass from an inflammatory one, that cannot be detected by ultrasonogram or CT scan^[8]. Finally intraductal ultrasound, as an adjunct to ERCP, can also be of help^[9]. Despite of all these modern diagnostic tools, the problem may become apparent only during surgery.

Surgical treatment for type I MS is partial cholecystectomy leaving the neck of the gallbladder in place^[10]. In some cases, open or laparoscopic total cholecystectomy may be performed^[11]. However some authors consider this a contraindication for laparoscopic cholecystectomy^[12-14].

Surgical treatment of type II MS is less clearly defined. Corlette and Bismuth^[15] have recommended partial cholecystectomy, oversuturing of the gallbladder cuff and insertion of a T-tube through the fistula as an adequate treatment for type II MS.

Choledochoplasty is an acceptable therapeutic approach but the amount of gallbladder tissue employed for this has not yet been standardized^[16].

Furthermore, cholecystoduodenostomy has been described^[17] and hepaticojejunectomy^[18] can also be used if complete destruction of the common hepatic duct occurs.

Reconstruction of the extrahepatic biliary tree in case of MS type II with bypass of the lesion using a Roux-

en-Y cholecysto-choledochal-jejunostomy as in our case can be carried out. To our knowledge, this is the first case described in the literature.

In conclusion, since the preoperative diagnosis of MS cannot be achieved, an awarded suspicion is necessary to avoid a lesion of the biliary tree if firm adherence around Carlot's triangle is found. The success of treatment is related to a precocious recognition of the condition even at the time of surgery when the individual characteristics of each case are considered^[18].

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CASE REPORT

Portal venous gas and thrombosis in a Chinese patient with fulminant Crohn's colitis: A case report with literature review

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Abstract

Ever since its earliest reports, portal venous gas (PVG) has been associated with numerous intraabdominal catastrophes and has served as an indication for urgent surgical exploration. It is traditionally regarded to be an ominous finding of impending death, with highest mortality reported in patients with underlying bowel ischemia. Today, computed tomography has demonstrated a wider range of clinical conditions associated with PVG, some of which are 'benign' and do not necessarily require surgery, unless when there are signs of intraabdominal catastrophe or systemic toxicity. One of these 'benign' conditions is Crohn's disease. The present report describes a 19-year-old Chinese boy with Crohn's pancolitis who presented with septic shock associated with PVG and portal vein thrombosis, and was successfully managed surgically. To our knowledge, this is the first report of PVG and portal vein thrombosis associated with Crohn's disease in a Chinese patient. In addition, we have also reviewed the reports of another 18 Crohn's patients with PVG previously described in the English literature. Specific predisposing factors for PVG were identified in 8 patients, including barium enema, colonoscopy, blunt abdominal trauma, and enterovenous fistula. The patients who developed PVG following barium enema and blunt trauma were all asymptomatic and no specific treatment was necessary. Eleven patients (58%) who presented with signs of intraabdominal catastrophe or systemic toxicity required either immediate or eventual surgery. The overall mortality rate among the 19 patients was only 11%. The present literature review has shown that the finding of PVG associated with Crohn's disease does not always mandate surgical intervention. It is the clinical features and the related complications that ultimately determine the treatment approaches. The overall outcome of PVG associated with Crohn's disease has been favourable.

INTRODUCTION

Portal venous gas (PVG) is a rare radiological finding that occurs when intraluminal gas from the gastrointestinal tract or gas-forming bacteria enters the portal venous circulation^[1]. Factors predisposing to PVG include bowel mucosal injury, bowel distension, and sepsis^[2]. Ever since its earliest reports^[3,4], PVG has been associated with numerous intraabdominal catastrophes and has served as an indication for urgent surgical exploration. It is traditionally regarded to be an ominous finding of impending death, with highest mortality reported in patients with underlying bowel ischemia^[2]. However, it is becoming apparent that there are conditions in which the finding of PVG is relatively 'benign' and does not always indicate surgery, and these conditions include digestive tract dilatation, ulcerative colitis, Crohn's disease, and complications of iatrogenic and endoscopic procedures^[5]. Remarkably, there are reports of PVG occurring in patients with uncomplicated Crohn's disease after blunt trauma^[6,7] or colonic diagnostic procedures^[8,9], which resolves spontaneously without treatment. On the other hand, when PVG occurs in Crohn's patients with signs of intraabdominal catastrophe or systemic toxicity, urgent surgery is warranted, and the final outcome has mostly been favourable^[10-18]. The present report describes a 19-year-old Chinese boy with Crohn's pancolitis who presented with septic shock associated with PVG and portal vein thrombosis, and was successfully managed surgically. To our knowledge, this is the first report of PVG and portal vein thrombosis associated with Crohn's disease in a Chinese patient. The literature of PVG associated with Crohn's disease is also reviewed, with special emphasis on the clinical features, the various predisposing factors, and the treatment approaches.

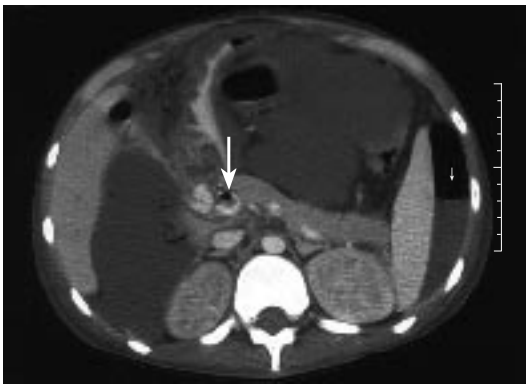


Figure 1 Computed tomography of the abdomen showing evidence of portal venous gas (long arrow), portal vein thrombosis, gross ascites, and pneumoperitoneum (short arrow).



Figure 2 Photograph of the total colectomy specimen showing the classical features of Crohn's colitis: cobblestone mucosa with skipped lesions.

CASE REPORT

A 19-year-old Chinese boy presented to the Accident and Emergency Department of our hospital with fever, hypotension, and abdominal distension. He had been diagnosed with Crohn's pancolitis a year ago and had been treated with mesalazine, but the drug compliance was poor. One month prior to this hospitalization, he had been admitted to another hospital because of fever and bloody diarrhoea. He was treated as a flare-up of Crohn's disease with intravenous steroid and antibiotics. However, his condition remained static despite a week of medical treatment, and he insisted to be discharged against medical advice. He consulted a Chinese herbalist and consumed some Chinese herbal medicines, but his condition continued to deteriorate. He became so ill that his parents finally brought him to our hospital to seek for further medical treatment.

On arrival, his temperature was 38°C, blood pressure 86/40 mmHg and heart rate 153 beats/min respectively. The abdomen was grossly distended and tense, although no frank peritoneal sign could be elicited. Initial blood tests showed leukocytosis, coagulopathy, and metabolic acidosis. He was immediately admitted to the Intensive Care Unit (ICU); aggressive fluid resuscitation and antibiotics were given. After initial stabilization, an urgent computed tomography (CT) of the abdomen and pelvis was performed, which showed evidence of portal venous gas, portal vein thrombosis, gross ascites, and pneumoperitoneum (Figure 1). The small bowel appeared thickened and inflamed, but the large bowels were not clearly demonstrated. The diagnosis was compatible with Crohn's disease with bowel perforation and septic shock, and hence an emergency laparotomy was arranged.

Intraoperatively, generalized peritonitis with 2–3 L of faeculent fluid and pus was noted throughout the peritoneal cavity. The small and large bowels were densely matted together by inflammatory adhesions. The large bowel appeared shrunken and chronically diseased, but no perforated site was identified. No obvious stricture or perforation was noted along the small bowel. The adhesions between the bowels were removed, and this already resulted in a blood loss of 9 L because of severe coagulopathy. Damage control surgery with packing of all the raw areas was done and the patient was sent back to the ICU for



Figure 3 Computed tomography of the abdomen showing evidence of partial recanalization of the portal vein (long arrow) with increasing surrounding collaterals (short arrow).

further stabilization. NovoSeven (recombinant coagulation factor VIIa) and blood products were given to correct the coagulopathy. A second-look laparotomy performed 24 h later showed no more active bleeding from the raw areas. No obvious perforated site was identified along the gastrointestinal tract. The small bowel appeared edematous but viable, while the large bowel appeared shrunken and unhealthy. Thus a total colectomy was performed to remove the diseased large bowel (Figure 2). The abdomen was temporarily closed using a sterile plastic bag to avoid abdominal compartment syndrome. The abdomen was subsequently closed at a 3rd laparotomy 48 h later and an end ileostomy was fashioned. Pathological examination of the resected colon showed severe Crohn's colitis with multiple ulcerations and deep fissures but without perforation.

The postoperative course was very stormy and the patient had prolonged stay in the ICU. Peritoneal swab cultures and blood cultures grew *Enterococcus* and Methicillin-resistant *Staphylococcus aureus* and so vancomycin was added to the treatment regimen. For the portal vein thrombosis, anticoagulation therapy was not started initially because of coagulopathy. Nevertheless, partial recanalization of the portal vein with increasing collaterals was evident on follow-up CT a few weeks later (Figure 3). One month after the 3rd surgery, he developed an episode of massive intraabdominal haemorrhage secondary to erosion of a

Table 1 Clinical features, predisposing factors, treatment, and outcome of 19 Crohn's patients with PVG reported in the English literature

Author (yr)	Sex/age	Clinical features	Predisposing factors	Diagnostic modality	Treatment	Operative findings	Outcome
Reiner <i>et al</i> (1978) ^[10]	F/34	Fever and abdominal pain	Enterovenous fistula	Plain X-ray	Antibiotics and surgery	Ileitis	Survived
Sadhu <i>et al</i> (1979) ^[8]	F/64	No symptom or morbidity	Barium enema	Plain X-ray	No treatment	/	Survived
Gosink (1981) ^[11]	M/45	Fever and abdominal pain	/	US	Surgery	Inflamed colon	Survived
Pappas <i>et al</i> (1984) ^[9]	M/36	No symptom or morbidity	Sigmoidoscopy, barium enema	Plain X-ray	No treatment		Survived
Huycke <i>et al</i> (1985) ^[12]	M/22	Resolution of toxic megacolon; developed abdominal pain and free peritoneal air after colonoscopy	Colonoscopy	Plain X-ray	Antibiotics and surgery	Ileitis and colitis, no perforation	Survived
Katz <i>et al</i> (1986) ^[21]	M/14	No symptom or morbidity	Barium enema	Plain X-ray	Antibiotics		Survived
Ajzen <i>et al</i> (1988) ^[13]	M/64	Severe epigastric pain	Enterovenous fistula	US, CT	Surgery	Ileitis	Died of sepsis and liver failure 1 mo later
Venugopal <i>et al</i> (1990) ^[14]	F/27	Fever	/	CT	Surgery	Ileitis, no perforated or ischaemic bowel	Survived
Kirsch <i>et al</i> (1990) ^[22]	F/26	Epigastric pain and chills	/	Plain X-ray, CT	Antibiotics	/	Survived
Delamarre <i>et al</i> (1991) ^[23]	M/70	Fever	/	CT	Antibiotics	/	Survived
al-Jahdali <i>et al</i> (1994) ^[15]	F/40	Fever	/	CT	Antibiotics (Surgery 2 wk later)	Ileitis	Survived
Hong <i>et al</i> (1997) ^[16]	M/58	Fever, status post-low anterior resection and small bowel resection	/	CT	Antibiotics	/	Survived
Hong <i>et al</i> (1997) ^[16]	F/71	Abdominal pain (Developed free peritoneal air 2 wk later)	/	CT	Antibiotics (Surgery 2 wk later)	Ischaemic and perforated small bowel	Died of disseminated cytomegalovirus infection
Hong <i>et al</i> (1997) ^[16]	M/24	Fever (Developed abdominal pain 4 wk later)	/	CT	Antibiotics (Surgery 4 wk later)	Ileitis	Survived
Brandon <i>et al</i> (2000) ^[17]	F/59	Fever and abdominal pain	/	CT	Surgery	Ileitis and colitis	Survived
Nesher <i>et al</i> (2002) ^[6]		No symptom or morbidity	Blunt trauma	CT	No treatment	/	Survived
Paran <i>et al</i> (2003) ^[7]	F/25	No symptom or morbidity	Blunt trauma	CT	No treatment	/	Survived
Thethy <i>et al</i> (2005) ^[18]	F/58	Fever	/	CT	Surgery	Sigmoid inflammatory mass	Survived
Present case	M/19	Septic shock with free peritoneal air	/	CT	Surgery	Colitis, no perforation	Survived

US: Ultrasonography; CT: Computed tomography.

mesenteric vessel by an infected collection and required a 4th laparotomy for haemostasis. Fortunately, he recovered quite uneventfully thereafter and was subsequently discharged home 2 mo after the first surgery.

The patient was put on maintenance mesalazine and azathioprine and remained well and asymptomatic 14 mo after the surgery. A CT enteroclysis was performed later and showed no evidence of small bowel involvement.

DISCUSSION

Portal venous gas was first described by Wolfe and Evans

in 1955 in 6 neonates with fatal necrotizing enterocolitis^[3]. In 1960, Susman and Senturia reported the similar finding in an adult, critically ill with small bowel infarction^[4]. Since then, PVG has been reported with increasing frequency in the literature. One of the first reviews by Liebman *et al* evaluated 64 patients with PVG on plain X-ray reported in the literature before 1977^[2]. According to that review, although PVG was observed in association with various clinical conditions, it was mostly (72%) found among patients seriously ill with necrotic bowel. The overall mortality was 75%, and this led the authors to recommend urgent surgical exploration for PVG except for patients with stable

ulcerative colitis who had undergone barium enemas, because 5 such patients survived with conservative treatment. Over the last 2 to 3 decades, advances in diagnostic radiology, including the development of ultrasonography and CT, have increased the sensitivity of imaging PVG. Brandon *et al* suggested that early recognition using CT, with appropriate surgical intervention, improves the chance for patient survival when PVG is identified^[17]. Moreover, PVG on CT has been found to be associated with a wider range of clinical conditions, some of which are 'benign' and do not necessarily require surgical intervention, especially when there are no signs of intraabdominal catastrophe or systemic toxicity^[19]. A recent study of 17 patients with PVG detected by CT has actually reported a mortality rate as low as 29%^[20]. Today, PVG is recognized as a mere diagnostic clue in patients with suspected acute abdominal pathology and is not itself a predictor of mortality. The more relevant prognosticator is the clinical condition in which PVG occurs.

One of the relatively 'benign' conditions that are associated with PVG is Crohn's disease. Including the present case, 19 cases of PVG associated with Crohn's disease were reported in the English literature (Table 1)^[6-18,21-23]. In 4 of these patients, PVG was iatrogenic in origin, resulting from barium enema^[8,9,21] or colonoscopy^[12]. Two patients developed PVG after blunt abdominal trauma^[6,7]. It has been postulated that elevated intraluminal pressure (during colonic diagnostic procedures) or intraperitoneal pressure (associated with blunt trauma) can permit bowel gas or gas-forming bacteria to gain access to the portal venous circulation through microscopic mucosal injury. Another factor predisposing to PVG is the development of entero-venous fistula between the bowel lumen and the mesenteric venous system, which is an extremely rare complication reported in 2 patients with Crohn's disease^[10,13]. In the remaining 11 patients, no specific predisposing factors could be identified. PVG in these patients is thought to be the result of mucosal injury and sepsis^[2] associated with bowel inflammation and portal pyaemia. The occurrence of PVG does not seem to be associated with the anatomical location of Crohn's disease involvement. Both ileal and colonic diseases can develop PVG.

The finding of PVG associated with Crohn's disease does not always mandate surgical intervention. It is the clinical features and the related complications that ultimately determine the treatment approaches. The presentation of PVG following blunt trauma^[6,7] and barium enema^[8,9,21] is remarkably innocuous; all the 5 reported patients were asymptomatic and no specific treatment was necessary (except in 1 patient who was given 'prophylactic antibiotics' for 48 h^[21]). Intravenous antibiotics were administered to patients who developed fever, and 3 of them had complete resolution of symptoms with this simple medical treatment^[16,22,23]. Eleven patients (58%) who presented with signs of intraabdominal catastrophe or systemic toxicity required either immediate or eventual surgery. All these patients underwent resection of the inflamed small and large bowels. The overall mortality rate among the 19 patients was only 11%; a patient with entero-venous fistula died of sepsis and liver failure 1 mo after surgery^[13], while another patient who underwent surgery for ischemic and perforated

small bowel finally died of disseminated cytomegalovirus infection^[16].

The combination of extensive pylephlebitis (or septic thrombosis of the portal vein) and PVG that occurs in our case is an extremely rare complication among patients with Crohn's disease; only 1 similar case was reported in the German literature^[24]. Severe active Crohn's disease and sepsis are the 2 predisposing factors^[25]. Although PVG itself is not a predictor of mortality, the finding of PVG combined with pylephlebitis is generally regarded as an ominous prognostic sign^[26]. Nevertheless, as illustrated in our case, favourable clinical outcome in these patients can still be achieved with early CT diagnosis, maximal organ support in the ICU, aggressive medical treatment, and prompt surgical intervention.

In conclusion, the finding of PVG associated with Crohn's disease does not always mandate surgical intervention. It is the clinical features and the related complications that ultimately determine the treatment approaches. The overall outcome of PVG associated with Crohn's disease has been favourable.

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Is there an alternative therapy to cyanoacrylate injection for safe and effective obliteration of bleeding gastric varices?

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TO THE EDITOR

We read with interest the article entitled "Bleeding gastric varices: Results of endoscopic injection with cyanoacrylate at King Chulalongkorn Memorial Hospital" by Noophun *et al*^[1]. They performed n-butyl-2-cyanoacrylate (CA) injection therapy for bleeding gastric varices in twenty-four patients, and hemostasis was achieved in seventeen (71%) patients. They concluded that CA injection therapy was effective and safe for bleeding gastric varices. However, we disagreed with the author's conclusion. Their hemostasis rate was relatively low, and two of the 24 patients developed serious complications as a result of glue embolism. Although CA injection therapy has been accepted as the first line treatment especially in Europe and Asia, hemostasis and obliteration of gastric varices are still a therapeutic challenge and many serious complications as a result of CA injection have been reported^[2]. In addition, there is no reimbursement from the insurance companies in Japan if CA is used for obliterating varices and therefore this therapeutic option is not widely practised there. Although conventional endoscopic injection sclerotherapy using ethanolamine oleate has been reported to be ineffective for bleeding gastric varices^[3], we conducted endoscopic injection sclerotherapy combined

with a vasoactive drug for bleeding gastric fundal varices. We have reported a series of thirty patients with bleeding gastric fundal varices treated with endoscopic injection sclerotherapy using 5% ethanolamine oleate plus infusion of vasopressin^[4]. With our method, continuous injection of 5% ethanolamine oleate mixed with contrast medium through a double lumen catheter was performed under fluoroscopic guidance until it filled the varices and their feeder veins, and thrombin glue was sprayed at the puncture site during withdrawal of the injector needle to prevent bleeding. We have achieved a high rate of hemostasis (93%) with a relatively low rebleeding rate (19%) over follow-up period of up to 5 years without any serious complications^[4]. We would like to recommend our sclerotherapy under fluoroscopic guidance method combined with medical therapy as an alternative to CA injection therapy for bleeding gastric varices, although randomized trials with a larger number of patients are warranted. If CA is to be used, aliquots of 1.0 mL CA per injection must be strictly enforced to prevent any embolic complications. Injections can be repeated if bleeding continues, as long as only 1.0 mL CA is injected each time^[5]. Fluoroscopic guidance might also be useful in minimizing this complication^[6].

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Meetings

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21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
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Vienna, Austria
Kenes International

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Immune response to *H pylori*

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Abstract

The gastric mucosa separates the underlying tissue from the vast array of antigens that traffic through the stomach lumen. While the extreme pH of this environment is essential in aiding the activation of enzymes and food digestion, it also renders the gastric epithelium free from bacterial colonization, with the exception of one important human pathogen, *H pylori*. This bacterium has developed mechanisms to survive the harsh environment of the stomach, actively move through the mucosal layer, attach to the epithelium, evade immune responses, and achieve persistent colonization. While a hallmark of this infection is a marked inflammatory response with the infiltration of various immune cells into the infected gastric mucosa, the host immune response is unable to clear the infection and may actually contribute to the associated pathogenesis. Here, we review the host responses involved during infection with *H pylori* and how they are influenced by this bacterium.

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Key words: *H pylori*; Immune response; T cell; Dendritic cells

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INTRODUCTION

H pylori is one of the most common human pathogens, since it infects the gastric mucosa of about 50% of the world's

population. The majority of infections are asymptomatic, making the infection lifelong without effective bacterial eradication. The clinical magnitude of this bacterium has become accepted rather recently. *H pylori* has been recognized as the causal agent for chronic gastritis and gastric and duodenal ulcers. Additionally, epidemiological and statistical studies associated the infection with a higher risk of gastric malignancy leading the World Health Organization International Agency for Research in Cancer to categorize *H pylori* as a class I carcinogen.

The bacteria induce a host immune response, but the persistence of the infection suggests that the response is not effective in eliminating the infection. Furthermore, multiple lines of evidence suggest that the immune response contributes to the pathogenesis associated with the infection. As a result, the immune response induced by *H pylori* is a subject of continuous study that has encouraged numerous questions. The inability of the host response to clear infections with *H pylori* could reflect down-regulatory mechanisms that limit the resulting immune responses to prevent harmful inflammation as a means to protect the host. Consequently, the chronic immune response induced may be inadequate or misdirected, and could thus afford a colonization advantage for the bacteria by providing improved availability of adhesion places. An example of this is the resulting increase in class II major histocompatibility complex (MHC) and CD74, induced by IFN- γ and IL-8, that are used as receptors by *H pylori*^[1-3].

H pylori has been shown to employ multiple mechanisms to antagonize, impair, or subvert host responses^[4]. For instance, *H pylori* has been noted to inhibit macrophage nitric oxide production and phagocytosis^[5]. Another mechanism where *H pylori* can down-regulate the immune response is through its VacA virulence factor. This cytotoxin can interfere with the processing and presentation of antigens by antigen-presenting cells (APCs)^[6], and can also inhibit T cell activation through interference of the calcineurin-associated IL-2 signaling pathway^[7]. These and multiple other observations on the nature of the immune response during *H pylori* infection have led to models that help explain how the bacteria could persist in the gastric environment by generating a non-effective immune response. The ineffective response, together with the host factors, determines the severity of the disease.

HUMORAL RESPONSE

Nearly everyone infected with *H pylori* develops specific antibodies, which are found in serum and in gastric

aspirates or extracts of stomach. Accordingly, elevated titers of IgG and IgA antibodies directed at membrane proteins (MP), flagelin, urease, lipopolysaccharide (LPS) and *H. pylori* adhesin A (HpaA) have been reported in patients infected with *H. pylori*^[8,9]. Yet, those titers do not differ between asymptomatic patients and patients with duodenal ulcers. IgM- and IgA-producing cells in biopsies from the antral region of the *H. pylori*-infected patients' stomachs were 40- to 50-fold higher in frequency than in non-infected subjects. However, IgG-producing cell numbers are the same for non-infected and infected *H. pylori* subjects. Those results suggest that the infection induces a large recruitment of immune cells into the gastric mucosa, particularly IgA-producing cells. A recent immunoproteome analysis compared individual sera from *H. pylori*-positive patients suffering from gastric adenocarcinoma or duodenal ulcer with a pool of five sera from *H. pylori*-negative patients to detect antigenic proteins from three separate *H. pylori* strains^[10]. That study recognized 30 antigens detected by *H. pylori* positive sera, nine of these were newly identified and 21 established previously. The study established the presence of antigens related to specific disease. Interestingly, cancer sera provided stronger immunoreactivity while a similar study suggested that sera from ulcer patients have more anti-*H. pylori* antibodies than sera from gastritis patients^[11].

Due to the plasticity of the *H. pylori* genome as well as the phase variation that the bacteria present in its LPS, specifically mimic Lewis antigens, 20% to 30% of the people infected with *H. pylori* develop autoantibodies, with most of them specific to the gastric proton pump located in the parietal cells. These antibodies may block pump function, leading to achlorhydria associated with the infection, which contributes to the gastric damage seen during infection.

T-CELL RESPONSE

H. pylori induce the recruitment of CD4⁺ and CD8⁺ T-cells into the gastric mucosa, but there appears to be preferential activation of CD4⁺ cells rather than CD8⁺ cells^[12]. Several studies have noted that the T helper cell response to *H. pylori* is polarized, since CD4⁺ T cells in the gastric mucosa of infected individuals produce the Th1 cytokines, interleukin (IL)-12 and interferon (IFN)- γ , whereas IL-4, a Th2 cytokine, production by these T cells is absent^[13,14]. A recent study by Amedei *et al*^[15] suggested that *H. pylori* neutrophil-activating protein (HP-NAP) contributes to this Th1-polarized T cell response in the gastric mucosa of *H. pylori*-infected patients. In that study, addition of HP-NAP to antigen-induced T cell lines in culture resulted in a shift from a predominant Th2 to a Th1 phenotype of specific T cells.

Another subset of CD4⁺ T cells are T regulatory cells, which produce IL-10 and transforming growth factor (TGF)- β ^[16]. While there is a demonstrated infiltration of T cells in the gastric mucosa and most of those are CD4⁺ T cells with markers of activation, various studies have tried to address the inefficiency of the host response in clearing the infection. Different studies have demonstrated that *H. pylori* infection can decrease T cell responses as well as

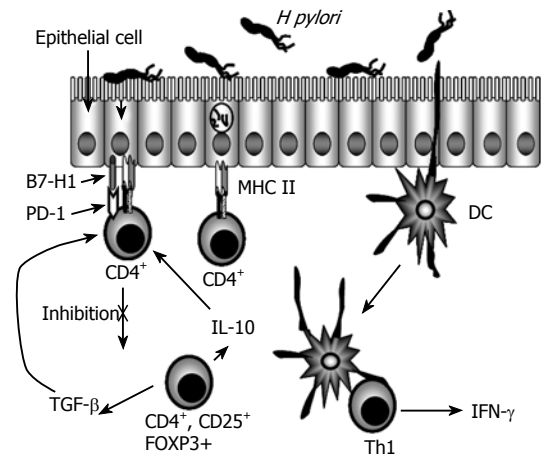


Figure 1 Regulation of CD4⁺ T Cells During *H. pylori* Infection. CD4⁺ T cell numbers increase in the gastric lamina propria of individuals infected with *H. pylori*. These cells are predominantly Th1 cells characterized by their production of IFN- γ . Because the epithelium separates *H. pylori* from CD4⁺ T cells, and also expresses key proteins associated with antigen presenting cells, the gastric epithelium, in addition to dendritic cells, could be involved in the presentation of antigens to these CD4⁺ T cells. The expression of inhibitory B7 related molecules along with CD4⁺ T cells with a regulatory T cell phenotype could be playing a role in limiting the function of effector CD4⁺ T cells.

induce T cell anergy^[12]. CD45RO⁺ memory T cells as well as activated CD69⁺ and CD25⁺ T cells are increased in the antral lamina propria of infected subjects^[17]. Memory T cells isolated from peripheral blood from infected people responded less to stimulation with *H. pylori* antigens than cells isolated from non-infected subjects^[12,18,19]. These results suggested the presence of regulatory T (T reg) cells, CD4⁺ and CD25⁺ in the peripheral blood of *H. pylori* infected individuals, which could inhibit the response of CD4⁺ T cells to *H. pylori*. This notion was supported by observations that a higher responsiveness was obtained after depletion of *H. pylori*-specific T reg cells^[20,21]. Hence, these observations may help explain the inability of the host response to eliminate the infection due to the activation of T reg cells, which were recently reported to be increased in the gastric mucosa of *H. pylori*-infected individuals and were described as CD4⁺, CD25^{high} and FOXP3⁺. Such cells may simultaneously reduce mucosal damage mediated by T cells as well as reduce specific T cell responses, possibly by reducing activation of IFN- γ -producing CD4⁺ T cells that can be effective in protection against the infection with these bacteria. Figure 1 illustrates the presence of the various T cell populations in the infected gastric mucosa and how they may interact with one another and with other resident cells.

The role of CD8⁺ T cells in the gastric mucosa of *H. pylori*-infected individuals is less clear than that of the CD4⁺ T cells. Although their numbers are also increased, the CD8⁺ T cells that are found in the infected tissue are thought to be intraepithelial lymphocytes. Their contribution to the local response is in the form of IFN- γ production, which in turn helps increase class II MHC molecule expression on adjacent cells. A recent report by Azem and colleagues showed that *H. pylori*-reactive CD8⁺ T cells can be efficiently stimulated by *H. pylori* antigen-pulsed B cells and DCs, and that most of the CD8⁺ T cells in the infected gastric mucosa are memory T cells^[21].

ANTIGEN PRESENTING CELLS IN THE GASTRIC MUCOSA

The activation of CD4⁺ T cells requires their effective cross talk with cells that express class II MHC molecules, which are classically referred to as antigen presenting cells (APC). Conventional APC include macrophages, dendritic cells and B cells. The role of these cells in the adaptive response is to internalize foreign antigens and present them in the form of peptides bound to class II MHC molecules to the T cells. The infected gastric mucosa contains a significant macrophage population that produces nitric oxide, IL-6, IL-1 β , TNF- α , and IL-12 that help drive a T helper 1 response responsible for the production of IFN- γ , and little or no IL-4 and IL-5^[22,23]. Although in smaller numbers, dendritic cells are also present and respond to *H pylori* with the production of IL-6, IL-8, IL-10 and IL-12, and have increased expression of CD80, CD83, CD86, and HLA-DR as a result of their stimulation with *H pylori*^[24].

For efficient T cell activation, T cells require not only the T cell antigen receptor (TCR)-mediated signaling, but also costimulatory signals provided by APC^[25]. The B7 family of molecules provides signals that are critical for both stimulating and inhibiting T cell activation. Engagement of CD28 by CD80 (B7-1) and CD86 (B7-2) stimulates and sustains T cell responses, whereas engagement of CTLA-4 by the same ligand inhibits T cell responses^[26]. Recently, several new members of the B7 family have been identified. B7-H2 (homologue 2 also known as GL50, B7h, B7RP-1 and LICOS) has been identified as a ligand for the CD28 family member ICOS (inducible T-cell co-stimulator). Two additional B7 family members, Programmed Death-Ligand 1, PD-L1 (B7-H1) and PD-L2 (B7-DC) bind to the receptor Programmed Death-1 (PD-1) and their interaction down regulates T cell activation^[27]. PD-1 is a type I transmembrane receptor expressed on activated T and B cells. Like CTLA-4, PD-1 contains an immunoreceptor tyrosine based inhibitory (ITIM) motif in its cytoplasmic region and acts as a negative regulator of lymphocyte function via multiple mechanisms, including cell-cycle inhibition and apoptosis. The literature suggests that there is another unidentified receptor for B7-H1 and B7-DC whose function has yet to be determined. Two other receptors of the B7 family are B7-H3 and B7-H4 (also known as B7S1 and B7x); however, their receptors and functions are still unclear^[28-30]. In an attempt to examine whether changes in the expression of these novel B7 family members could contribute to the hyporesponsiveness of T cells in the infected gastric mucosa, we examined by real-time PCR the expression of the message for these molecules in gastric biopsies and observed the expression of B7-H1, B7-DC, and B7-H3. Since, as discussed below, the epithelium is exposed to both *H pylori* and T cells in the lamina propria, we examined the epithelium for these molecules and detected their expression by PCR and by Western blot analysis. B7-H1 was the most prominent coinhibitory molecule of the B7 family whose expression was induced following *H pylori* infection. More interesting, epithelial cells in gastric biopsies infected with *H pylori* showed higher B7-H1

expression compared with uninfected samples^[31]. Gastric epithelial cells were found to constitutively express B7-H1, and the level of expression increased significantly during infection. T cells cocultured with gastric epithelial cells exposed to *H pylori* had a lower proliferation index, IL-2 secretion, and CD69 expression in response to activation via CD3. However, blockage of B7-H1 with specific anti-B7-H1 antibodies restored the responses to levels close to those of T cells not cocultured with gastric epithelial cells. This may represent a novel mechanism of immune avoidance used by *H pylori*, which involves the induction of coinhibitory molecule expression on gastric epithelial cells by the bacterium.

Recent elegant studies by Anderson and colleagues showed the importance of CTLA-4 in establishing T cell anergy during *H pylori* infection in a murine model. In this model of *H pylori* infection, the mice that received anti-CTLA-4 Fabs responded to an *H pylori* challenge with much greater inflammation and drastically decreased bacterial numbers. Their results suggested that CTLA-4 engagement may represent yet another mechanism of inactivation of *H pylori*-specific T cells during *H pylori* infection, which could in turn contribute to the chronicity of this infection^[32].

While direct interaction between APC and T cells represent the traditional mechanism leading to T cell activation, another mechanism that is under active investigation involves exosomes secreted by APC. Exosomes are small membrane vesicles derived from late endosomes, which are released into the extracellular membrane and interact with membranes of other cells at a relative distance. Exosomes secreted by APC carry class I and II MHC molecules, costimulatory molecules, and adhesins. Thus, they have immunomodulatory capacity, such as in the activation of naïve T cells^[33]. They have been shown to stimulate T cells *in vitro* and to induce anti-tumor responses *in vivo*^[34,35]. While they are not yet characterized in the context of the T cell response to *H pylori*, their contribution in modulating the local response has to be considered.

Human dendritic cells have been shown to produce IL-8, IL-10, and IL-12 in response to *H pylori* as well as to purified *H pylori* antigens^[36,37]. Thus, *H pylori* can bind to the dendritic cell receptor DC-specific ICAM-3-grabbing nonintegrin (SIGN) through the blood group Lewis X antigen present in its LPS^[38]. This interaction can alter the T helper balance and favor pathogen persistence. Also, in monocytes, urease and HSP60 have been shown to be potent activators of proinflammatory cytokines via NF- κ B activation^[39,40].

THE GASTRIC EPITHELIUM AS AN ACTIVE PLAYER IN THE MUCOSAL RESPONSE

In terms of providing protection, the gastric epithelium has typically been regarded as a physical barrier; however, multiple studies have provided evidence to suggest that the gastric epithelium plays a key role in the inflammatory and immune responses induced by *H pylori*. The epithelium is the only cell phenotype in the gastric mucosa that is in direct contact with the pathogen. This feature places the

epithelium in a strategic situation to interact with *H pylori* and with the immune elements in the lamina propria. There is strong evidence to suggest that the gastric epithelium is an active player in the response while performing functions associated with antigen presenting cells^[41,42]. In addition, it is well documented that the epithelium has the ability to produce cytokines that trigger the recruitment of inflammatory cells into the gastric lamina propria^[1]. The production of IL-8 in response to *H pylori* infection is one of the first epithelial responses. This chemokine recruits immunological components into the gastric mucosa from the periphery, particularly polymorphonuclear cells, which contribute to epithelial damage^[43]. Macrophages also contribute to epithelial damage by producing nitric oxide in response to *H pylori* urease leading to the induction of additional inflammatory mediators^[44]. However, the bacteria produce an arginase encoded by the gene *rocF* that competes with the NOS for L-arginine and converts this to urea and L-ornithine rather than NO^[45].

One of the major mechanisms of IL-8 induction by epithelial cells is through the injection of CagA into gastric epithelial cells by a type IV secretion system^[46]. This system releases CagA into the epithelial cells cytosol inducing cell proliferation and IL-8 production^[47]. Our group has recently described the interaction of *H pylori* with CD74 on gastric epithelial cells (GEC) leading to the production of IL-8, *via* NF- κ B activation^[1]. Interestingly, IL-8 induced by *H pylori*, in addition to its effect in the recruitment of inflammatory cells, also acts in an autocrine manner and induces further expression of CD74^[2]. This, in turn, suggests that *H pylori* has the ability to induce the increased expression of receptors on the host epithelium to enhance colonization and the stimulation of proinflammatory responses. As part of the inflammatory response, we noted that the *H pylori*-infected gastric epithelial cells produce macrophage migration inhibitory factor (MIF), which is an important cytokine that bridges the innate and adaptive immune responses^[48]. The production of MIF was found to be dependent on CagA, since CagA-deficient mutant *H pylori* strains had a significantly reduced ability to stimulate MIF production.

Some of the interactions of the epithelium with *H pylori* can be detrimental to the integrity of the epithelium. For instance, we have shown that *H pylori* use class II MHC as receptors on GECs, and this interaction leads to apoptosis^[3]. This interaction is mediated *via* *H pylori* urease. It has also been reported that *cag* genes may up-regulate Fas ligand (FasL) expression leading T cells to undergo apoptosis^[49]. Thus, the contribution of the gastric epithelium in influencing the adaptive response by expressing molecules that either directly or indirectly limit T cell activity has to be considered in our ongoing efforts to understand the host response to *H pylori*.

THE INNATE RESPONSE TO *H PYLORI*

Other potential interactions that lead to production of pro-inflammatory cytokines include that of *H pylori* with toll-like receptors (TLR) expressed by epithelial cells. It has been reported that gastric epithelial cells express TLR2, TLR4, TLR5, and TLR9^[50-53] that

interact respectively with lipoproteins, LPS, flagellin, and CpG motifs. The expression of those receptors by epithelial cells is of importance in innate immunity against *H pylori*. Since these innate receptors may elicit cytokine secretion when they bind their ligands, they may have an indirect effect in the subsequent adaptive response through the enhancement of processing and presentation of antigen by host cells. However, it has been demonstrated that *H pylori* LPS has a 500-1000 fold lower endotoxin activity than LPS from *S. typhimurium* and *E. coli*^[54,55]. This low stimulatory potential can be attributed to the phosphorylation pattern and the LPS' lipid A acylation^[55]. In addition, *H pylori* LPS has low binding affinity to LPS-binding protein (LBP) and in consequence, has a lower transfer rate to CD14 present in macrophages and monocytes^[56].

Another ligand for TLR receptors on the epithelium is *H pylori* flagellin. This flagellin contains different amino acids than that of other bacteria in the TLR5 recognition site, as well as having a compensatory mutation that preserves bacterial motility. Those differences avoid the recognition of flagellin by TLR5^[57]. *H pylori* also avoids recognition by TLR9, which is the receptor for unmethylated CpG motif present in bacteria and viruses. Since *H pylori* DNA shows a high rate of methylation, it can evade the recognition of its DNA by TLR9.

Mast cells represent another innate cell phenotype that is found within the *H pylori*-infected gastric mucosa of humans and mice^[58]. These cells represent an innate defense component that may kill bacteria through the release of proteases and other mediators. Additionally, an interesting observation was made in a recent study that showed that these cells can mediate bacterial clearance in vaccinated mice, and were suggested to do so *via* a cross talk with CD4⁺ T cells^[59].

In parallel with CagA, peptidoglycan (PGN) is also translocated into the epithelial cells by the *cag pathogenicity island* (PAI)-encoded type IV secretion system. Cag-PAI positive bacteria can induce the production of IL-8 *via* NF- κ B in a manner that is CagA-independent by signaling through Nod1. Thus, *H pylori* PGN can interact with Nod1 and induce the activation of NF- κ B^[60].

CONCLUDING REMARKS

Infection with *H pylori* results in robust innate and acquired immune responses by the host, where the gastric epithelium represents a central player. Interaction of *H pylori* with the host epithelium results in the release of an array of chemokines and cytokines. Some of these factors are stimulated via the engagement of toll-like receptors or cell surface receptors, such as CD74. Also, injection of CagA via the bacterial type IV secretion system leads to NF- κ B activation and the ensuing release of cytokines. The infected gastric mucosa is infiltrated by neutrophils and mononuclear cells as well as components of the acquired response, such as lymphocytes. A specific humoral response is also triggered during infection, as well as a T cell response that is skewed toward a Th1 cell response. In spite of these immune mechanisms, *H pylori* is not cleared because the bacteria seem to be equipped

with an array of mechanisms that allows them to evade or downregulate the host responses. Understanding these multiple mechanisms is a required step toward the development of any immune intervention strategies to protect from initial infection and to eliminate infections that are already established.

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H. pylori and host interactions that influence pathogenesis

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Abstract

H. pylori is probably the most prevalent human pathogen worldwide. Since it was initially suggested in 1983 by Marshall and Warren to be implicated in gastritis and peptic ulcer disease, *H. pylori* has also been implicated in gastric carcinoma and was classified as a class I carcinogen. In the last two decades, a noteworthy body of research has revealed the multiple processes that this gram negative bacterium activates to cause gastroduodenal disease in humans. Most infections are acquired early in life and may persist for the life of the individual. While infected individuals mount an inflammatory response that becomes chronic, along with a detectable adaptive immune response, these responses are ineffective in clearing the infection. *H. pylori* has unique features that allow it to reside within the harsh conditions of the gastric environment, and also to evade the host immune response. In this review, we discuss the various virulence factors expressed by this bacterium and how they interact with the host epithelium to influence pathogenesis.

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Key words: *H. pylori*; Gastric cancer; Immune response; Vacuolating cytotoxin

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H. PYLORI INFECTION AND DISEASES ASSOCIATED WITH THE INFECTION

H. pylori is one of the most common pathogens affecting humankind, infecting approximately 50% of the world's population. This pathogen is a gram-negative spiral shaped bacterium that has the unique ability to colonize the human gastric mucosa. The infection is usually acquired early in life and may persist a lifetime, unless treated. Of those infected, many will develop asymptomatic gastritis, but 10% develop gastric or duodenal ulcers, and approximately 1% develop gastric carcinoma. The outcome of the infection may involve a combination of the bacterial factors, host factors, as well as environmental factors. Ulceration and carcinogenesis are mutually exclusive outcomes of this infection. *H. pylori* infection is a very persistent infection, and in areas of high prevalence, reinfection is also very common.

A very high percentage of gastric and duodenal ulcers (up to 85%) are attributable to *H. pylori* infection. Patients in the United States who are infected with *H. pylori* have a 3.5 times increased risk of developing peptic ulcer disease than uninfected persons^[1]. A hallmark feature of infection with *H. pylori* is a pronounced inflammatory response and the inability of the host to clear the infection, which results in a persistent infection, increased acid production, and tissue damage.

It is now well accepted that chronic infection with *H. pylori* is a major risk factor in the development of gastric cancer. *H. pylori* has been shown to induce changes in the gastric mucosa that could contribute to the development of cancer. Given the strength of the evidence supporting an association between adenocarcinomas of the gastric mucosa and *H. pylori* infection, *H. pylori* has become classified as a class I carcinogen by the International Agency for Research on Cancer in affiliation with the World Health Organization^[2]. Gastric cancer remains the second deadliest cancer worldwide. On a global scale, gastric cancer accounts for approximately 700 000 deaths annually. In the US there are 24 000 new cases and 14 000 deaths annually^[3].

Infection with *H. pylori* also plays a critical role in the development of mucosa-associated lymphoid tissue (MALT) lymphoma. *H. pylori* is present in the gastric mucosa of most cases of MALT lymphoma, and 75% of these cases regress after eradication of *H. pylori*^[4,5]. Interestingly, gastric MALT lymphoma is the only known

Table 1 *H pylori* adhesins and the gastric epithelial receptor for each

Adhesin	Receptor
BabA	Lewis B blood group antigen
SabA/B	Sialyl Lewis X
HpaA	Sialyl Lewis X
UreB	CD74
UreA?	Class II MHC
AlpA/B	?
HopZ	?
?	DAF
?	Sulfated molecules (heparan sulfate)
?	Phospholipids
?	Trefoil Factor 1

malignancy whose course can be directly changed by the removal of a pathogen. Thus, *H pylori*-associated diseases are a significant global problem and result in considerable morbidity, mortality, and societal costs.

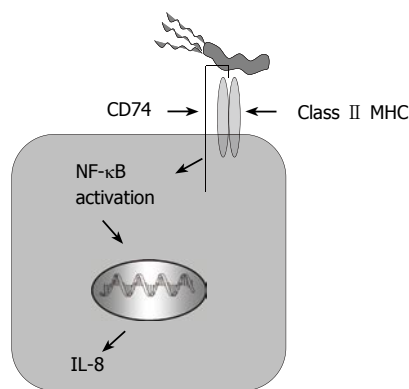
H pylori virulence factors and pathogenesis

H pylori colonizes the gastric epithelial apical surface, but the precise mechanisms of adherence and pathogenesis are still being elucidated. Adherent strains are able to survive in the gastric mucosa, colonize at high densities, and are able to re-colonize, while non-adherent strains are readily removed^[6]. Thus, adhesion is crucial in the ability of *H pylori* to persist and cause disease. In addition to contributing to colonization, adherence results in signal transduction, activation of NF- κ B and subsequent secretion of interleukin-8, which is important in the inflammatory response during infection.

Adhesins

An assortment of molecules on gastric epithelial cells (GECs) have been proposed as receptors for *H pylori* adherence, as well as multiple adhesins that have been identified on the outer membrane of *H pylori*, but those responsible for pathogenic events are still being investigated (Table 1). Several well known adhesins are BabA, SabA, and AlpAB. BabA and SabA bind to fucosylated and sialylated blood group antigens, respectively. There are clearly multiple adhesins and receptors for *H pylori* because only half of the strains in the U.S. have detectable BabA^[7]. While the attachment of *H pylori* using BabA as an adhesin does not appear to induce signaling or immune responses from host cells, SabA appears to be required for activation of neutrophils and the resulting oxidative burst by binding to sialylated neutrophil receptors^[8]. Although the AlpAB receptor is unknown, it may be even more important as an adhesin because studies with knockout strains dramatically reduced adherence of the bacteria to some cells^[9]. HopZ, another adhesin being investigated, also showed decreased adherence when a knockout strain was utilized^[10], but not as dramatically as the AlpAB knockout strain. *H pylori* urease can also act as a bacterial adhesin^[11]. Urease present on the bacterial surface due to bacterial lysis or release^[12,13] binds to class II MHC molecules on host cells, and may induce their apoptosis^[11].

Other studies from this group also suggest that the

**Figure 1** *H pylori* binds to CD74 on gastric epithelial cells and induces NF- κ B activation and IL-8 production.

urease B subunit binds to CD74, which is expressed in polarized fashion on the luminal side of the epithelium^[14], and in doing so stimulates gastric epithelial IL-8 release^[15] (Figure 1). A recent study by O'Brien and colleagues showed that, similar to other pathogens, *H pylori* uses decay accelerating factor (DAF aka CD55) as a receptor for binding to the gastric epithelium^[16]. Since there have been multiple *H pylori* adhesins described, bacterial adhesion is clearly a complex mechanism with multiple outcomes depending on the host cell receptor engaged.

Cytotoxicity associated pathogenicity island

Following colonization and attachment, various virulence factors expressed by certain *H pylori* strains appear to promote disease. For instance, the expression of *cagA* and *vacA* genes by strains of *H pylori* is highly associated with disease^[17]. These *cagA* gene-expressing strains have also been associated with peptic ulcer and patients infected with these strains have an increased risk of gastric cancer^[18]. The *cagA* gene is considered a marker for a cluster of genes referred to as pathogenicity island (PAI). *cagPAI* is known to encode for a type IV secretion system that allows CagA, and possibly peptidoglycan, to be delivered into epithelial cells (Figure 2). CagA is tyrosine phosphorylated by Src family kinases^[19], and has differing numbers of tyrosine phosphorylation motifs (EPIYA motifs), which determine the virulence of the *H pylori* strain and host cell response to it. The amount of EPIYA motifs is directly related to the levels of phosphorylation and cytoskeletal rearrangement seen in epithelial cells^[20]. Phosphorylated CagA interacts with a protein tyrosine phosphatase, SHP-2 inducing its phosphatase activity. Upon activation of SHP-2, it is able to induce host cells signaling, such as MAP kinase/MEK/ERK1/2 signaling through Ras/Raf. Dysregulation in this pathway is responsible for increased cell proliferation and moving of gastric epithelial cells (cell spreading) and cell elongation (hummingbird phenotype)^[21]. Interaction of CagA with other signaling molecules such as growth factor receptor-binding protein-2 (Grb-2), hepatocyte growth factor scatter factor receptor (c-Met), and phospholipase C gamma (PLC- γ) can induce similar phenotypes in gastric epithelial cells^[22,23]. Phosphorylated CagA inhibits the activity of Src kinases in a negative feedback loop^[19]. Thus, the inhibition on Src kinases activity also results in dephosphorylation of a set

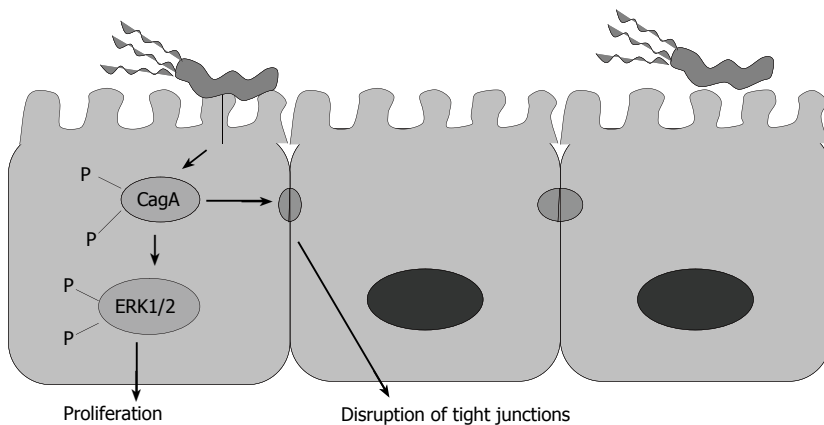


Figure 2 CagA is injected into the cell by a type IV secretion system where it is phosphorylated and induces ERK1/2 phosphorylation and increased cell proliferation. CagA also induces disruption of the tight junctions between adjacent cells.

of host cell proteins, including the actin-binding protein cortactin. In parallel to ERK1/2 signaling, NF- κ B may also be activated, and upregulation of pro-inflammatory cytokines may also ensue^[24]. While it was initially thought that the CagA injection was not responsible for the production of proinflammatory cytokines, but other proteins of the *cagPAI* were thought to play a crucial role, it has more recently been shown that CagA injection into the host gastric epithelial cell can induce NF- κ B activation and IL-8 production^[24]. In fact, independent studies showed that transfection of *cagA* into gastric epithelial cells induced IL-8 production^[25]. The activation of the Ras/MEK/ERK pathway occurs following the interaction of CagA with Grb2. Because CagA interacts with important signaling mediators in the host cells, it is considered responsible for changes in cell morphology, adhesion and turnover. CagA also induces the recruitment of ZO-1 and junctional adhesion molecule (JAM) to the site of bacterial attachment causing disruption of the epithelial barrier and dysplastic alterations in epithelial cell morphology. This effect is phosphorylation independent^[26]. The delivery of *H. pylori* peptidoglycan via the *cagPAI*-encoded type IV secretion system results in the intracellular binding of peptidoglycan by Nod1, an intracellular pathogen-recognition molecule, and this may also contribute to the induction of gastric epithelial responses^[27]. The *H. pylori* Type IV secretion system (T4SS) stimulates the Rho family GTPases Rac1 and Cdc42 in gastric epithelial cells in an independent CagA translocation mechanism^[28]. Thus, Rac1 and Cdc42 are recruited to the membrane at sites of infection activating p21-activated kinase (PAK1) and rearrangements of the cytoskeleton^[29]. The T4SS can also activate the receptor tyrosine kinase, epidermal growth factor receptor (EGFR), regulating the ERK1/2 pathway *via* Ras phosphorylation^[30]. Furthermore, CagA can inhibit B-cell proliferation by suppressing the JAK-STAT signaling pathway. Hence CagA, in a phosphorylation-independent pathway, can diminish the immune response against *H. pylori* and play a role in the development of MALT lymphoma by impairing p53-dependent apoptosis pathway^[31].

Vacuolating cytotoxin

The vacuolating toxin, or VacA, is a pore forming toxin that has the ability to induce vacuole formation in cells and disrupt normal membrane trafficking. VacA, is expressed

by about half of all *H. pylori* strains. Like CagA, VacA appears to be unique to *H. pylori* since no other species have a homologue. VacA has effects on many cell types, including gastric epithelial cells, antigen presenting cells, mast cells, and lymphocytes, which makes it an important virulence factor. This toxin is secreted by *H. pylori*, and it binds to the plasma membrane of host cells where it forms anion-selective channels. The receptors for VacA are EGFR and RPTP- α and - β ^[32]. However, VacA also binds to detergent-resistant microdomains (lipid rafts) and GPI-APs^[33,34]. This process results in the release of nutrients from the cell, and the bacteria may use these for survival^[35]. As the anion concentration becomes higher inside the cell through these pores, proton pumping also increases, as does an influx of weak bases. The weak bases are protonated and trapped inside, causing osmotic swelling, and the formation of a vacuole^[36]. VacA can also disrupt mitochondrial membrane potential and affect cellular ATP concentrations, which disrupt the cell cycle progression and lead to apoptosis^[37,38]. Another significant way in which VacA contributes to pathogenesis is by inhibiting the processing of antigens by B-cells and their presentation to CD4+ T-cells^[39], as well as the T cell activation and proliferation. When mixed with T cells, VacA suppresses NFAT^[40,41,42], IL-2 production, and surface expression of IL-2 receptors, which are required for T cell proliferation and viability^[36]. Multiple signaling pathways of T cell activation are also affected by VacA exposure, which is one mechanism *H. pylori* may use to evade immune responses. In gastric epithelial cells, VacA activates the p38 and ERK-1/2 MAP kinases, thereby contributing to the induction of immune responses by these cells^[43]. In fact, the *vacA* gene product has been shown to cause in mice, some of the tissue damage observed in *H. pylori*-infected patients^[44].

Urease

Another major virulence factor of *H. pylori* is urease, which is expressed by all strains. Urease is composed of two subunits, α , which is approximately 24 kDa, and β , which is approximately 68 kDa. *H. pylori* produces a large amount of urease, representing 5% to 10% of the total protein content of the bacteria. This enzyme is essential for the survival and pathogenesis of the bacteria. Perhaps the most important role urease plays is to hydrolyze urea into CO₂ and NH₃, which aids in buffering the bacteria from

the acidic conditions it may encounter in the stomach. Urease is crucial for *H pylori* colonization, as shown by studies where urease negative strains were not able to colonize in multiple animal studies^[45,46]. The inability of urease negative strains to colonize was initially assumed to be due to their inability to buffer their niche. However, similar studies under hypoacidic conditions also led to the same results, where urease negative mutants of *H pylori* could not colonize in an animal model. These observations suggested a role for urease beyond its enzymatic function. Although much urease is located intracellularly, there is some present on the bacterial surface^[47,13]. *H pylori* surface-associated urease can act as an adhesin for the bacteria, which induces the production of inflammatory cytokines from both gastric epithelial cells and macrophages^[48,49], along with apoptosis of some cells^[11]. While the mechanism of action associated with these responses elevated by urease is not entirely clear, the induction of apoptosis may result as a consequence of binding to class II MHC^[50]. The urease B subunit can also bind to CD74 and induce IL-8 production by gastric epithelial cells^[51]. Both of these responses are important in the overall pathogenesis seen during *H pylori* infection.

Other factors

Another important disease-associated virulence factor of *H pylori* is the outer inflammatory protein A (OipA). OipA is part of a family of 32 outer membrane proteins characterized as part of the *H pylori* genome. This protein has been suggested to induce pro-inflammatory responses from gastric epithelial cell lines. In one study with *H pylori* clinical isolates, those isolates expressing OipA, but not the *cag* pathogenicity island proteins were able to induce IL-8 production from gastric epithelial cell lines at 3 times the level of strains that did not express either^[52]. Isolates from Japan all expressed OipA, while isolates from the U.S. did not, and thus it is thought that the presence of OipA may make Japanese strains more virulent. When the signaling induced by *cag*PAI proteins was compared to OipA, OipA was found to induce phosphorylation of Stat1, while the *cag*PAI proteins induced NF- κ B activation^[52]. Both of these signaling pathways contribute to induction of IL-8 production, but act in conjunction with one another to fully activate the IL-8 promoter.

Some other virulence factors expressed by *H pylori* include neutrophil-activating protein (NAP) and heat shock protein 60 (Hsp60)^[53,54]. NAP has been shown to be a chemoattractant for both monocytes and neutrophils during *H pylori* infection^[55]. Hsp60 has been shown to induce proinflammatory cytokines by macrophages and gastric epithelial cells^[56,57], which appears to be mediated by Toll-like receptors (TLRs). Lipopolysaccharide expressed by *H pylori* is a very weak immunogen compared to that of other gram negative bacteria, but it has been shown to induce some proinflammatory cytokines^[58]. Although the interactions that initiate epithelial cell signals following bacterial adherence are critical in pathogenesis, they are not well understood, nor are the responses of the gastric epithelium that contribute to chronicity of the infection.

In a VacA paralogue mechanism *H pylori* can secrete collagenase, which can degrade collagen present in the

extracellular matrix to supply the bacteria with essential amino acids. During chronic infections, collagenase can exacerbate ulcer development and deter the process of ulcer healing^[59].

VIRULENCE FACTORS AND INFLAMMATION

Perhaps the most important response for the pathogenesis associated with infection is inflammation. Both antigen specific and non-specific responses contribute to inflammation during infection. These responses contribute to fighting infection, but are also responsible for mucosal damage. Adhesion of *H pylori* to the host epithelium, or bacterial factors such as urease or the *cag*PAI proteins, induce signaling that upregulates proinflammatory cytokines and chemokines such as IL-8 and GRO- α . In a recent study, we showed that CagA is important in the induction of macrophage migration inhibitory factor (MIF) production by the gastric epithelium^[60]. This cytokine has significant importance in the innate and adaptive host responses. Although the *cag*PAI proteins are the most recognized factor inducing inflammatory responses, there are several other interactions known to upregulate these responses. We have recently discovered that through *H pylori* attachment to CD74, or crosslinking CD74 on gastric epithelial cells, NF- κ B activation occurs leading to IL-8 production^[15]. Blocking this interaction with monoclonal antibodies resulted in a substantial decrease in the amount of IL-8 produced in response to *H pylori*. Other bacterial factors that induce inflammatory responses are HSP60, which was shown to induce IL-8 through Toll-like receptor pathways^[57], and urease, which induced responses from both gastric epithelial cells and peripheral blood mononuclear cells^[49]. The cytokine responses, in turn, recruit other immune cells to the site of infection such as IL-8, which is one of the initial chemokines that recruits neutrophils to the site of infection. Neutrophils are then activated by *H pylori* or its soluble products, and proceed to release reactive oxygen species (ROS) and more IL-8^[61], which lead to tissue damage associated with infection.

THE IMPACT OF *H PYLORI* ON THE GASTRIC EPITHELIUM

Cell turnover rate

Another response of GECs to infection is enhanced proliferation. In order to balance the increased growth of epithelial cells, the host must compensate by increasing epithelial cell turnover. One mechanism to account for epithelial cell turnover is increased cell death. Apoptosis provides a highly regulated mechanism for cell loss in both healthy and inflamed tissue. In the digestive tract, apoptosis has been described as being important in the control of normal epithelial cell turnover while it is increased in the gastric epithelium during infection with *H pylori*^[62,63]. The rate of apoptosis induction may be regulated by exogenous cytokines and growth factors. For example, we have shown that IFN- γ can directly augment the ability of *H pylori* to induce apoptosis of GECs^[50]. An indirect effect of IFN- γ is its ability to induce an increased expression of putative receptors (i.e., class II MHC and CD74) for *H pylori*. Our

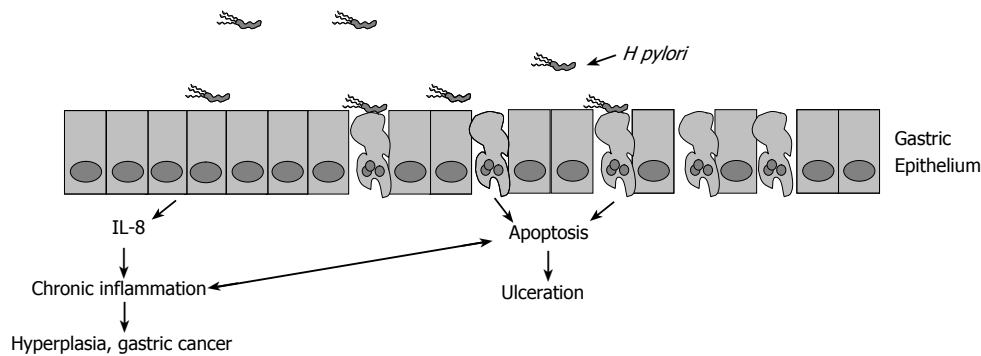


Figure 3 *H. pylori* induces chronic inflammation leading to either ulceration or malignant outgrowths.

studies have shown that *H. pylori* induces the expression of class II MHC and CD74^[50,64], and another study showed that *H. pylori* induces yet another receptor, sialyl-Le^x, on GEC^[65].

Other cytokines and chemokines induced by *H. pylori* may also play a role in inducing GEC proliferation. MIF, which is produced by GEC in response to CagA injection^[60], and may be produced by macrophages and T cells during infection^[66] can induce GEC proliferation. MIF affects proliferation by inactivating p53 tumor suppressor gene and inducing proliferative signaling such as ERK1/2 activation. Additionally, IL-8 has recently been shown to promote GEC proliferation by accelerating the processing of EGFR ligands, which bind and induce transactivation of the receptor^[67]. In one study administering nonsteroidal anti-inflammatory drugs was capable of decreasing GEC proliferation in the mouse model^[68], further suggesting a role for pro-inflammatory cytokines in cell turnover during *H. pylori* infection.

ROS and DNA damage

The prevalence of IL-8 produced by the gastric epithelium at the site of infection results in the infiltration of neutrophils. *H. pylori* soluble factors activate neutrophils^[69], which go on to produce reactive oxygen species (ROS). Gastric epithelial cells have also been shown to produce ROS in response to *H. pylori*^[70]. Interestingly, *H. pylori* appear to be resistant to the antimicrobial action of ROS. However, ROS may also induce DNA damage in the epithelium, which leads to apoptosis. Since ROS production appears to be dependant on bacterial load^[71], it also may be correlated to the amount of damage to the epithelium. When there is a lower bacterial load, the balance between oxidants and antioxidants in the gastric mucosa is disrupted at levels not high enough to induce apoptosis. The risk of DNA damage from ROS is high, and thereby may lead to pro-carcinogenic events.

Overview of the impact of *H. pylori* on pathogenesis

The interactions of *H. pylori* with the host are a complex series of events that induce pathogenesis while allowing the bacterium to persist. Only about 20% of the bacteria are bound to the epithelium *via* multiple adhesions at any given time^[72]. Attachment to the epithelium, along with multiple virulence factors, induce proinflammatory immune responses. These responses can affect host cell viability and lead to one of two mutually exclusive events. Either excess gastric acid is produced leading to ulceration,

or chronic inflammation induces atrophy of the stomach wall and malignant outgrowths (Figure 3). The unique and persistent interactions of *H. pylori* with the host, along with 50% worldwide infection rates, make it a significant pathogen that induces considerable disease.

SUMMARY

While a significant volume of knowledge has been acquired during the last decade to help us understand how *H. pylori* causes disease, there is still no available vaccine that is effective against this pathogen. *H. pylori*'s ability to maintain its long-term residence in a broad segment of humankind is in large part due to the subversion of common structures on the host cells for its interactions. As with most infectious agents, adhesion to host cells is a crucial step in colonization. Attachment is facilitated by various structures or adhesins on the bacteria which include BabA, SabA, HopZ, AlpA/B, and urease. These adhesins bind to carbohydrate moieties on blood group antigens, as is the case of BabA and SabA binding to Lewis antigen, or to proteins of central importance to the host response, such as urease binding to class II MHC and CD74. These interactions are important to characterize in detail as they may offer insights into novel prophylactic or therapeutic agents directed at *H. pylori*-associated diseases.

Following colonization, *H. pylori* employs virulence factors, such as the cagPAI type IV secretion system and a vacuolating cytotoxin to exert damage on the host epithelium and to alter the host immune response. The ability of VacA to disrupt endosomal traffic and thus alter antigen presentation, together with its ability to arrest T cell cycle progression, makes VacA an important virulence factor that could contribute to the establishment of chronic infection. As we understand the mechanisms that contribute to the long term residence of *H. pylori* in the human stomach, we will be better able to prevent the chronicity that underlies the development of the serious diseases associated with this infection.

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EDITORIAL

Interleukin-12 and Th1 immune response in Crohn's disease: Pathogenetic relevance and therapeutic implication

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Abstract

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory disorders of the gastrointestinal tract that share clinical and pathological characteristics. The most accredited hypothesis is that both CD and UC result from a deregulated mucosal immune response to normal constituents of the gut microflora. Evidence, however, indicates that the main pathological processes in these two diseases are distinct. In CD, the tissue-damaging inflammatory reaction is driven by activated type 1 helper T-cell (Th1), whereas a humoral response predominates in UC. Consistently, a marked accumulation of macrophages making interleukin (IL)-12, the major Th1-inducing factor, is seen in CD but not in UC mucosa. Preliminary studies also indicate that administration of a monoclonal antibody blocking the IL-12/p40 subunit can be useful to induce and maintain clinical remission in CD patients. Notably, the recently described IL-23 shares the p40 subunit with IL-12, raising the possibility that the clinical benefit of the anti-IL-12/p40 antibody in CD may also be due to the neutralization of IL-23 activity. This review summarizes the current information on the expression and functional role of IL-12 and IL-12-associated signaling pathways both in patients with CD and experimental models of colitis, thus emphasizing major differences between IL-12 and IL-23 activity on the development of intestinal inflammation.

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Key words: Interleukin-12; Type 1 helper T-cell cytokines; Inflammatory bowel disease

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INTRODUCTION

Inflammatory bowel disease (IBD) is the general term indicating Crohn's disease (CD) and ulcerative colitis (UC), two chronic inflammatory disorders of the intestine that have different morphological, immunological and clinical characteristics. The etiology of IBD is unknown, but evidence has been accumulated to show that the liability to develop CD or UC is influenced by a wide range of genetic and environmental factors, which have been only in part characterized. Over the last recent years, it has also become evident that both CD and UC are caused by excessive immune reactivity in the gut wall, and that this is directed against normal constituents of the luminal flora. However, CD and UC are immunologically different diseases, even though they share end-stage effector pathways of tissue damage. These advances led to the development of novel therapeutic agents that are currently being studied for their capacity to specifically target the mucosal inflammatory pathways occurring in IBD patients.

IL-12 AND TH1 CYTOKINES IN CD

In both CD and UC, the inflamed tissue is heavily infiltrated with leukocytes, mostly T lymphocytes. These cells are activated and make increased amounts of cytokines, which are thought to have a primary role in promoting the disease process. Using sensitive assays, several authors have shown that CD and UC have distinct profiles of cytokine production. While in CD there is a predominant synthesis of type 1 helper T-cell (Th1) cytokines, including IFN- γ and TNF- α , Th2 cytokines, such as IL-5 and IL-13, are considered to have a more prominent role in UC^[1,2]. T-lamina propria lymphocytes (T-LPL) isolated from the inflamed colon of UC patients also make more IFN- γ than normal T-LPL following *in vitro* activation with anti-CD3/CD28 antibodies^[1]. Therefore, the classic Th1-Th2 paradigm seems to be overly simplistic, and there is now sufficient evidence to believe that these two pathways can co-exist rather than being mutually exclusive in the human gut.

The discovery that IFN- γ -secreting T-LPL are abundant in CD mucosa has paved the way for studies in which the switch that controls the differentiation of such cell type was investigated. This research led to the demonstration that in CD mucosa there is increased production of IL-12, the major Th1-inducing factor in man^[3,4]. IL-12 is a heterodimeric cytokine composed

of two covalently linked subunits (p40 and p35) and synthesized by monocytes/macrophages/dendritic cells^[5]. Transcripts for both IL-12 subunits have been detected in gastric and intestinal mucosa of patients with CD^[3,6]. In addition, it was shown that lamina propria mononuclear cells isolated from intestinal mucosal areas of CD, but not UC, patients released *in vitro* functionally active IL-12, and that neutralization of endogenous IL-12, in CD mucosal cell cultures, resulted in a significant decrease in the number of IFN- γ -producing cells^[3,4].

IL-12 mediates its biological activities through a receptor composed of two subunits, $\beta 1$ and $\beta 2$ ^[5]. Although both subunits are required to form a functional receptor, $\beta 2$ appears to be crucial in controlling Th1 cell lineage commitment^[7,8]. Consistently, high expression of IL-12R $\beta 2$ has been described in various Th1-mediated diseases, as well as in CD T-lamina propria lymphocytes (T-LPL)^[9-11]. Additionally, CD mucosal lymphocytes express high levels of active STAT-4, a transcription factor that is activated by IL-12R signals and is necessary to promote the induction of IL-12-driven Th1-associated genes^[11]. Notably, T cells from STAT-4-deficient mice manifest impaired IFN- γ production in response to IL-12 and are unable to efficiently promote the development of colitis when transferred in immunodeficient mice^[12]. On the other hand, studies in mice over-expressing STAT-4 revealed that such animals developed colitis that is characterized by the presence of a diffuse infiltration of Th1 cytokine-secreting cells in the intestinal wall^[13].

While IL-12 appears to be sufficient to trigger the Th1 cell program in naïve T cells, the expansion and maintenance of Th1 cell response in the gut would require additional signals (Figure 1). Indeed, the IL-12-induced synthesis of IFN- γ by intestinal lamina propria T lymphocytes can be enhanced by cytokines that signal through the common γ -chain receptor, such as IL-7, IL-15 and IL-21^[14,15]. Additionally, in CD mucosa, there is an enhanced production of biologically active IL-18, a cytokine involved in perpetuating Th1 cell responses^[16,17]. Immunohistochemical analysis has localized IL-18 to both lamina propria mononuclear cells and intestinal epithelial cells. In these cells, the expression of IL-18 is invariably associated with active subunits of IL-1 β -converting enzyme, a molecule capable of cleaving the precursor form of IL-18 to the active protein^[16,17]. Moreover, functional studies showed that down-regulation of IL-18 expression in cultures of CD lamina propria mononuclear cells by specific IL-18 antisense oligonucleotides significantly inhibited IFN- γ synthesis, further supporting the concept that IL-18 serves as a strong costimulatory factor of IL-12-driven Th1 responses^[16]. A newly discovered TNF-superfamily cytokine (TL1A) has also been involved in initiating or promoting the Th1 response in CD as well as in experimental models of IBD^[18,19]. Another protein that could contribute to the ongoing Th1 immune response in CD is osteopontin, a 60 kDa phosphoprotein, that is highly expressed in epithelial cells and macrophages in CD and shown to increase IL-12 production in CD mucosal cells^[20].

An analysis of transcription factors expressed in Th1 vs Th2 cells led to the discovery of T-bet, a novel

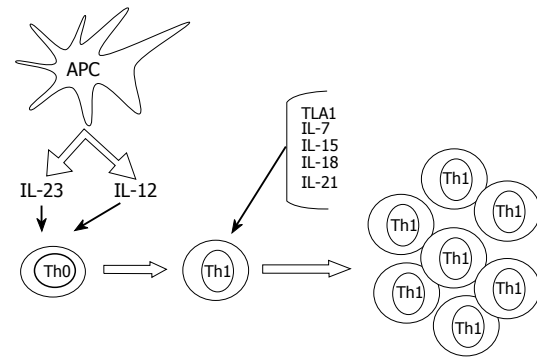


Figure 1 Some putative mechanisms implicated in the induction and expansion of Th1 cells in the gut of patients with Crohn's disease. Cytokines produced by antigen presenting cells, such as IL-23 and IL-12, promote the differentiation of Th1 cells. The expansion and mucosal accumulation of this cell subtype are then sustained by additional molecules, such as IL-18, IL-7, IL-15, IL-21 and TL1A.

member of the T-box family of transcription factors. T-bet drives chromatin remodelling of the IFN- γ locus and up-regulates IL-12R $\beta 2$ chain. Therefore, its expression strictly correlates with the differentiation of Th1 cells^[21]. As expected, T-bet is markedly over-expressed in CD4+ T-LPL of patients with CD and it associates with a reduced expression of GATA-3, a transcription factor that governs Th2 cell polarization^[22].

The molecular mechanisms underlying T-bet induction are not fully understood, even though there is evidence that cytokines that activate STAT1, such as IFN- γ and IL-21, may positively regulate T-bet expression. In line with these observations, neutralization of IL-21 in cultures of CD mucosal T cells is followed by a decreased expression of T-bet and secretion of IFN- γ ^[15].

REGULATION OF IL-12 PRODUCTION

A critical question remains as to what induces IL-12 in CD gut and which mechanisms are in place to regulate IL-12 production. IL-12 is produced by antigen-presenting cells mostly in response to bacteria or bacterial products/components^[5]. Since the development of Th1-mediated colitis both in humans and mice requires the presence of gut microbiota, it is conceivable that IL-12 production is driven by luminal bacteria. Indeed, it was shown that LPMC isolated from the inflamed intestine of CD patients are hyper-responsive to sonicates of bacteria from autologous intestine (BsA), and this phenomenon associates with increased expression of activation markers on both CD4+ and CD8+ lymphocyte subsets and production of IL-12 and IFN- γ ^[23]. Consistently, both local and systemic tolerance to BsA is broken in a murine model of chronic intestinal inflammation induced by the hapten reagent 2, 4, 6-trinitrobenzene sulfonic acid (TNBS), which mimics several important characteristics of CD. Tolerance to BsA is, however, restored in mice systemically treated with antibodies to IL-12^[24].

The reason why CD LPMC would respond to luminal bacteria with enhanced production of IL-12 remains, however, unclear. One possibility is that, in CD, LPMC are primed to synthesize high levels of IL-12 by specific stimuli. This hypothesis is suggested by the recent

observation that flagellin, a major antigenic target of immune response associated with CD^[25], can activate innate immunity *via* Toll-like receptor 5 (TLR5), and instruct dendritic cells to promote Th1 responses *via* IL-12p70 production^[26]. Another possibility is that CD LPMC lack negative regulators of bacteria-driven intracellular signals, and therefore would respond to bacterial stimulation with enhanced IL-12 synthesis. In line with this, it has recently been shown that splenocytes of mice carrying on deletions of CARD15, a gene whose mutations are associated with CD, and encoding NOD2, respond to peptidoglycan (PGN) stimulation with exaggerated activation of NF- κ B and production of IL-12 and IL-18^[27]. According to these data, NOD2 would function as a negative regulator of IL-12 production mediated by PGN. Therefore, in the absence of this negative regulation, PGN could elicit an excessive NF- κ B-dependent IL-12 response by mucosal cells^[28]. In the gut, NOD2 also regulates the production of anti-bacterial peptides, such as defensin-5, by Paneth cells^[29]. Consistently, epithelial cells expressing mutated NOD2 have a reduced capacity to restrict proliferation of bacterial pathogens in monolayer cultures^[30], raising the possibility that CARD15 mutations could facilitate the colonization of the intestine with bacteria that eventually sustain macrophage/dendritic cell activation and enhance IL-12-driven Th1 cell responses. Whether this explanation really fits with the mucosal IL-12 synthesis in CD patients with mutations remains, however, unknown, as no study has yet analyzed whether intestinal mucosal cells of CD patients with CARD15 mutations make *in vivo* more IL-12 than those of CD patients without CARD15 mutations.

IL-12-INDUCED T CELL RESPONSE LEADS TO MUCOSAL DESTRUCTION IN HUMAN FETAL GUT

Taken together, the above data suggest that the IL-12-driven Th1 signaling pathway can be important in immune-mediated injury in the gut. This is also substantiated by observations made in *ex vivo* models of T cell-mediated gut inflammation. By using human fetal gut explants, we previously showed that activation of T-LPL by anti-CD3 and IL-12 resulted in a strongly Th1-biased response that was followed by severe tissue injury, with destruction of the mucosa. Furthermore, analysis of explants culture supernatants revealed that stimulation of fetal gut tissues with anti-CD3 and IL-12 increased the production of matrix metalloproteinase 1 (MMP-1, collagenase) and MMP-3 (stromelysin 1), while the synthesis of tissue inhibitors of MMP-1 and 2 remained unchanged^[31]. Stromelysin 1 has a broad substrate specificity, being capable of degrading proteoglycans, laminin, fibronectin, collagen core protein, and non-helical cross-linked regions of type IV collagen. Stromelysin 1 has, therefore, the potential to destroy the structure of the intestinal lamina propria, thereby removing the scaffolding on which the epithelium lies. Indeed, abundant stromelysin 1 has been found in the mucosa of patients with CD, particularly near ulcers^[32]. Notably, the addition of a stromelysin 1 inhibitor to the IL-12-stimulated fetal gut organ culture prevented

the tissue damage without altering T cell activation. Similarly, a p55 TNF receptor human IgG fusion protein was able to prevent the mucosal degradation and inhibit stromelysin 1 production, thus suggesting that TNF- α is a key mediator of the IL-12-induced tissue damage^[31].

BLOCKADE OF IL-12 FACILITATES THE RESOLUTION OF TH1-MEDIATED INFLAMMATION IN THE GUT

The role of IL-12 in the mucosal inflammation in patients with CD is also supported by the demonstration that this cytokine is produced in excess in experimental models of Th1-induced colitis, such as the TNBS-induced colitis. Importantly, treatment of mice with antibodies to IL-12/p40 abrogates the TNBS-induced colitis, and the beneficial effect of such a treatment has been linked to the capacity of the blocking antibody to enhance mucosal T cell apoptosis through a FAS-dependent mechanism^[33,34].

Consistently, a randomized controlled study of 79 CD patients receiving 1 or 3 mg of an anti-IL-12p40 monoclonal antibody *versus* placebo demonstrated a response in 75% of CD patients compared with 25% in the placebo group. These responses paralleled a decrease in downstream cytokines, including IFN- γ and TNF- α ^[35]. In subsequent studies, it was also shown that patients with CD manifested both increased IL-12p70 and IL-23 secretion before anti-IL-12p40 mAb treatment and normal levels of secretion of these cytokines following cessation of treatment. Moreover, IL-23-induced T cell production of IL-17 and IL-6 was greatly reduced after IL-12 antibody treatment^[36]. More recently, it has been shown that treatment of active CD patients with two doses of Fontolizumab, an anti-IFN- γ antibody, which interferes with Th1 polarization as well as activation of macrophages, monocytes and natural killer cells, resulted in increased rates of clinical response and induction of remission compared with placebo^[37].

THE EMERGING ROLE OF IL-23 IN GUT INFLAMMATION

Whereas the central role of IL-12 in the generation of IFN- γ -secreting cells has long been appreciated, recent studies have shown that Th1 cell responses can also be regulated by IL-23. Importantly, IL-23 shares with IL-12 the p40 subunit^[38], and therefore, IL-23 biological activity is fully inhibitable by neutralising IL-12p40 antibodies. This fact and the demonstration that IL-23 is up-regulated in CD mucosa^[39] raise the question whether the beneficial effect of the blocking IL-12p40 antibody observed in CD patients is due to the neutralization of IL-12 and/or IL-23. Results from studies in mice with targeted deletion of either the IL-12/p35 or IL-23/p19 subunit suggest the possibility that IL-23 and not IL-12 is essential for manifestation of intestinal inflammation occurring in IL-10-deficient mice. The IL-23-driven intestinal inflammation appears to be mediated by the production of IL-17 and IL-6. Moreover, administration of recombinant IL-23 acceler-

ates the development of colitis in lymphocyte-deficient recombina-activating genes-knockout (RAG-KO) mice after reconstitution with CD4⁺ T cells from interleukin-10-knockout (IL-10-KO) mice^[40]. Whether IL-23 plays a similar role in other models of Th1-mediated colitis, such as the TNBS-colitis, remains however unknown. Similarly, it remains to be ascertained whether the deleterious effect of IL-23 on the ongoing mucosal inflammation occurs only in the absence of IL-10-related regulatory effects.

In conclusion, human IBD are thought to be caused by a dysregulated T cell response directed against constituents of the intestinal bacterial microflora. In CD, such a response is associated with an exaggerated production of IL-12 and IFN- γ . There is also evidence that the recently described IL-23 may drive the intestinal inflammation in murine models of IBD, thus suggesting that strategies aimed at specifically inhibiting the p19 subunit of IL-23 could be therapeutically useful in CD. Some observations made in cell systems, however, suggest to be cautious before drawing any conclusion. In fact, it has been reported that T-bet negatively regulates IL-17 production, thus promoting the shift of IL-17-producing cells towards a classical Th1 phenotype characterized by high IFN- γ ^[41]. Based on these findings, it is conceivable that IL-23 may play a determinant role in the early phase of T cell-mediated immune responses, thus leaving the place to IL-12/IFN- γ /T-bet pathway in the late phase.

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Upper gastrointestinal function and glycemic control in diabetes mellitus

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Abstract

Recent evidence has highlighted the impact of glycemic control on the incidence and progression of diabetic micro- and macrovascular complications, and on cardiovascular risk in the non-diabetic population. Postprandial blood glucose concentrations make a major contribution to overall glycemic control, and are determined in part by upper gastrointestinal function. Conversely, poor glycemic control has an acute, reversible effect on gastrointestinal motility. Insights into the mechanisms by which the gut contributes to glycemia have given rise to a number of novel dietary and pharmacological strategies designed to lower postprandial blood glucose concentrations.

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Key words: Blood glucose; Diabetes mellitus; Gastric Emptying; Gastrointestinal motility; Hyperglycemia

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INTRODUCTION

Diabetes and its long-term complications, which include cardiovascular, renal, neurologic, and ophthalmic disease, represent a major cause of morbidity and mortality throughout the world^[1]. The prevalence of both type 1 (insulin-dependant) and type 2 (non insulin-dependant) diabetes is increasing, the latter as a consequence of obesity. In the US, 29 million people and 14% of adults have diabetes or impaired fasting glucose, of whom about a third are undiagnosed^[2]. Similar figures are evident throughout the developed world^[3].

Hyperglycemia is central to the pathogenesis of diabetic micro- and macrovascular complications^[4]. There is increasing evidence that postprandial hyperglycemia is the major determinant of “average” glycemic control, and represents an independent risk factor for macrovascular disease, even in people without diabetes^[5]. While the relative importance of individual determinants of the blood glucose response to a meal remain to be clarified precisely, it is clear that upper gastrointestinal motility has a major impact that has generally been overlooked. Moreover, postprandial glycemic control in turn has a profound effect on the motor function of the upper gut. Hence, blood glucose concentration is determined by, as well as a determinant of, gastric and small intestinal motility^[6].

The aims of this review are to examine (1) evidence relating to the importance of postprandial, as opposed to fasting, glycemia in the development and progression of diabetic complications, (2) the contribution of upper gastrointestinal function to postprandial blood glucose concentrations, (3) the impact of variations in glycemia on gastric and small intestinal motility, and (4) the therapeutic strategies suggested by these insights.

IMPACT OF POSTPRANDIAL GLYCEMIA ON COMPLICATIONS OF DIABETES

The DCCT/EDIC and UKPDS trials established that the onset and progression of microvascular, and probably macrovascular complications of diabetes, are related to “average” glycemic control, as assessed by glycated hemoglobin^[4,7,8]. This provides a rationale for the widespread use of intensive therapy directed at the normalisation of glycemia. In the recently reported DCCT/EDIC study, a period of intensive, as opposed to conventional, therapy for a period of 6.5 years between 1983 and 1993 was shown to be associated with a reduction in the risk of a subsequent cardiovascular event by 42%^[4]. Glycated hemoglobin is potentially influenced by both fasting and postprandial blood glucose concentrations. However, given that the stomach empties ingested nutrients at a closely regulated overall rate of 2-3 kcal per minute^[9,10] and humans ingest around 2500 kcal daily, it is clear that most individuals spend the majority of each day in either the postprandial or post-absorptive phase with a limited duration of true “fasting” for perhaps three or four hours before breakfast^[11]. Hence, the traditional focus on the control of “fasting” blood glucose in diabetes management appears inappropriate.

It is well established that postprandial hyperglycemia precedes elevation of fasting blood glucose in the

evolution of diabetes^[12]. Furthermore, it appears to be the better predictor of coronary artery^[13] and cerebrovascular^[14] complications, and predicts all-cause and cardiovascular mortality, even in the general population without known diabetes^[15]. Blood glucose values at two hours during an oral glucose tolerance test are a better predictor of mortality than fasting blood glucose^[16], and reduction in cardiovascular risk in patients with type 2 diabetes is associated with control of postprandial, as opposed to fasting, glycemia^[17]. For example, patients with impaired glucose tolerance who were treated with the α -glucosidase inhibitor acarbose, to reduce postprandial glycemia, experienced a reduction in cardiovascular risk of about a third compared to placebo during a mean of three years follow-up^[18]. Postprandial blood glucose concentrations correlate well with glycated hemoglobin in the setting of mild to moderate hyperglycemia^[19], with fasting blood glucose only assuming greater importance at higher HbA1c values^[20]. There is also evidence that therapy directed towards lowering postprandial blood glucose concentrations may have a greater impact on HbA1c than attention to fasting blood glucose^[21].

Hyperglycemia potentially has diverse effects on blood vessels. In the short term, hyperglycemia is associated with activation of protein kinase C, which affects endothelial permeability, cell adhesion, and proliferation in the vessel wall. Over the longer term, non-enzymatic glycosylation of proteins results in atherosclerosis^[22]. Elevated postprandial blood glucose concentrations are associated with an increase in plasma biochemical markers of oxidative stress^[23,24]. However, to what degree hyperglycemia *per se* accounts for this effect, as opposed to concurrent elevations of non-esterified fatty acids and triglycerides, remains to be elucidated^[25].

CONTRIBUTION OF UPPER GASTROINTESTINAL FUNCTION TO POSTPRANDIAL GLYCEMIA

Postprandial blood glucose levels are potentially affected by a number of factors, including pre-prandial blood glucose concentration, food properties such as viscosity, fibre content, and quantity and type of carbohydrate, gastric emptying, small intestinal delivery and absorption of nutrients, insulin secretion, hepatic glucose metabolism, and peripheral insulin sensitivity^[26]. The relative importance of these factors is likely to vary with time after a meal, and between healthy subjects and patients with type 1 or type 2 diabetes. While it is logical that the gastrointestinal tract, which controls the rate at which ingested carbohydrate is absorbed and releases peptides that stimulate insulin secretion, should have a major impact on postprandial glycemia, its role has frequently been overlooked and generally underestimated in the past. The rate of gastric emptying is now established as a major contributor to variations in glycemia, while the influence of small intestinal motor function represents a current research focus.

Gastric emptying

Gastric emptying accounts for at least 35% of the variance

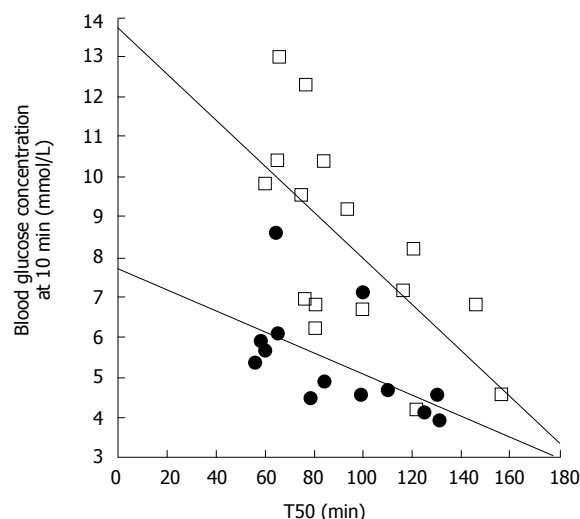


Figure 1 Relationship between the blood glucose concentration 10 min after consuming 75 g glucose in 300 mL water, and the gastric half-emptying time (T50) in patients with type 2 diabetes (open squares, $r = -0.67$, $P < 0.005$) and healthy subjects (filled circles, $r = -0.56$, $P < 0.05$). Adapted from Jones *et al* 1996^[28].

in the initial rise as well as the peak blood glucose levels after an oral glucose load in both healthy individuals^[27] and patients with type 2 diabetes^[28] (Figure 1). Pharmacological slowing of gastric emptying by morphine reduces the postprandial glycemic response to a mixed meal in type 2 patients, whilst acceleration of gastric emptying by erythromycin (a motilin agonist) increases postprandial blood glucose concentrations (Figure 2). Here, differences in peak blood glucose values are more marked than those in the area under the blood glucose curve^[29]. In type 1 patients the glycemic response to a meal, and therefore the requirement for exogenous insulin, is also critically dependent on the rate of gastric emptying. Here, when emptying is slower, the insulin requirement to achieve euglycemia is less^[30].

In health, gastric emptying is modulated by feedback arising from the interaction of nutrients with the small intestine, so that the overall rate of gastric emptying is closely regulated at about 2-3 kcal per minute^[9]. Infusion of a caloric load directly into the small intestine slows gastric emptying by a mechanism that includes relaxation of the gastric fundus, suppression of antral motility, and stimulation of phasic and tonic pressures in the pylorus^[31,32]; the latter acts as a brake to gastric outflow. The length of small intestine exposed to nutrient appears to be the primary determinant of the magnitude of the feedback response^[33,34]. Both transection of duodenal intramural nerves^[35], and suppression of the release of small intestinal peptides by the somatostatin agonist, octreotide^[36], accelerate the emptying of nutrient liquids, indicating the involvement of both neural and humoral mechanisms in mediating feedback from the small intestine. Glucagon like peptide-1 (GLP-1), which suppresses antral and duodenal motility and stimulates pyloric contractions^[37-39], probably represents one such humoral mediator, and the slowing of gastric emptying by this peptide appears likely to be the major mechanism by which its administration improves postprandial glycemia in patients with type 2 diabetes^[40]. While the contribution of endogenous GLP-1 in regulating gastric emptying

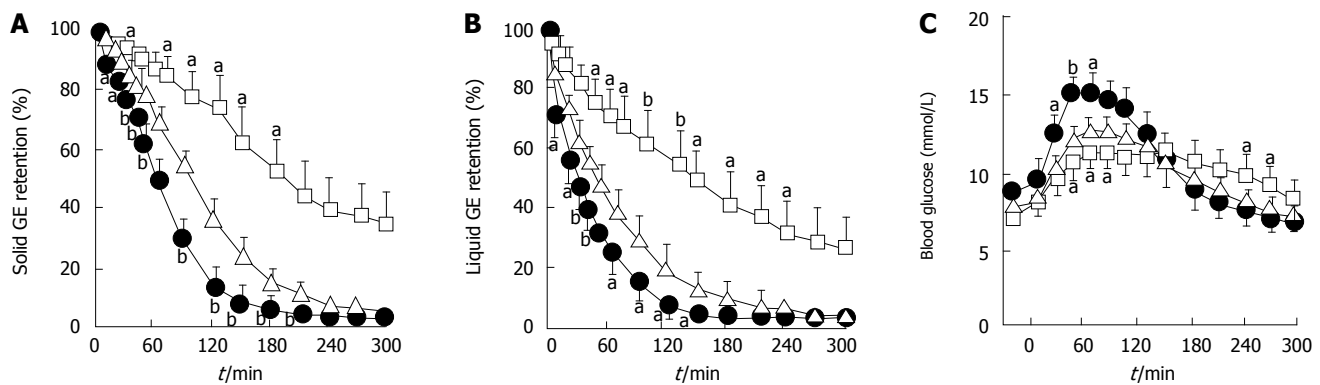


Figure 2 Effects of erythromycin (200 mg iv, filled circles) and morphine (8 mg iv, open squares) compared to placebo (open triangles) on (A) solid and (B) liquid gastric emptying and (C) blood glucose concentration in 9 patients with type 2 diabetes. ^a $P < 0.05$, ^b $P < 0.01$ vs placebo. Adapted from Gonlachanvit *et al* 2003^[29].

remains to be clarified (i.e. the “physiological” as opposed to “pharmacological” action), a recent study using the GLP-1 antagonist, exendin (9-39) amide, confirmed that endogenous GLP-1 mediates the suppression of antral motility and stimulation of pyloric pressures induced by the presence of glucose in the small intestine^[38].

It has long been recognised that oral or enteral administration of glucose results in a much greater insulin response than an equivalent intravenous glucose load^[41-44], a phenomenon referred to as the “incretin” effect. The putative incretin peptides, GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), are released from the small intestine in response to nutrients^[45], apparently in a load-dependent fashion^[46]. Therefore, the rate of delivery of carbohydrate from the stomach into the small intestine is likely to be critical in determining not only the rate of glucose absorption, but also the incretin response. Although GIP is the more potent of the two hormones in healthy individuals^[47], the insulinotropic effect of GIP appears to be markedly diminished in patients with type 2 diabetes, while the insulin response to GLP-1 is retained^[45]. This forms a rationale for the therapeutic use of GLP-1 and its analogs in the management of type 2 diabetes (to be discussed). There is limited evidence that type 2 diabetes is associated with an impaired GLP-1 response to oral glucose^[48], but to what degree delayed gastric emptying, which occurs frequently in type 2 patients^[49], accounts for this decrease in GLP-1 has not been evaluated.

In considering the potential impact of gastric emptying on postprandial glycemia, the initial rate of glucose entry into the small intestine (“early phase” of gastric emptying) may be particularly important^[50]. Type 2 diabetes is characterised by a reduced “early”, and frequently increased “late”, postprandial insulin response. Studies in rodents have established the importance of “early” insulin release as a determinant of postprandial glucose excursions, in that a small “early” increase in portal vein/peripheral blood insulin is more effective than a larger, “later” increase at reducing blood glucose levels^[51]. A recent study involving both healthy subjects and type 2 patients, established that modest physiological variations in the initial rate of small intestinal glucose entry have major effects on the subsequent glycemic, insulin and incretin responses (Figure 3)^[52]. Nevertheless, while initially rapid, and subsequently slower, duodenal glucose delivery can boost incretin and insulin responses when compared to

constant delivery of an identical glucose load, the overall glycemic excursion is, if anything, greater^[52], and certainly not improved^[53]. This evidence adds to the rationale for the use of dietary and pharmacological strategies designed to reduce postprandial glycemic excursions by slowing gastric emptying, rather than initially accelerating it. However, the “dose-response” relationship between duodenal glucose delivery and glycemia remains to be clearly determined.

Small intestinal glucose absorption

The gut lumen is the site of absorption of glucose from the external environment into the body, as well as being the source of the incretin peptides that drive much of the postprandial insulin response. Thus, it is logical that variations in small intestinal function should be a major determinant of postprandial glycemia. Nevertheless, there is little information about the impact of small intestinal motility and absorptive function on glycemia, at least in part because of the technical demands in studying this region of gastrointestinal tract^[6,54].

The large surface area of the small intestine is well suited to absorption of water and solutes. Perfusion studies in healthy humans have established that the proximal jejunum has a maximal absorptive capacity for glucose of approximately 0.5 g per minute per 30 cm^[55-57]. Small intestinal mucosal hypertrophy occurs in animal models of diabetes, with concomitant increases in glucose absorption, but this is rapidly reversed by insulin treatment^[58]. However, acute hyperglycemia does result in transient increases in intestinal glucose absorption in rodents^[59-61]. The few studies performed in humans with type 1^[62] “insulin-requiring”^[63], or type 2^[64] diabetes have not demonstrated increased small intestinal glucose absorption, other than one report of increased absorption at high luminal glucose concentrations^[65]. Attention was paid to maintaining euglycemia in at least one of these studies^[63]. However, there is a recent report of increased expression of monosaccharide transporters in humans with type 2 diabetes^[66], the clinical significance of which remains to be clarified. One human study failed to demonstrate an effect of marked hyperglycemia (14 mmol/L) on jejunal glucose absorption in healthy subjects^[63], although a relationship has been observed between more physiological postprandial blood glucose concentrations (less than 10 mmol/L) and the absorption of the glucose analog, 3-O-methylglucose, in healthy

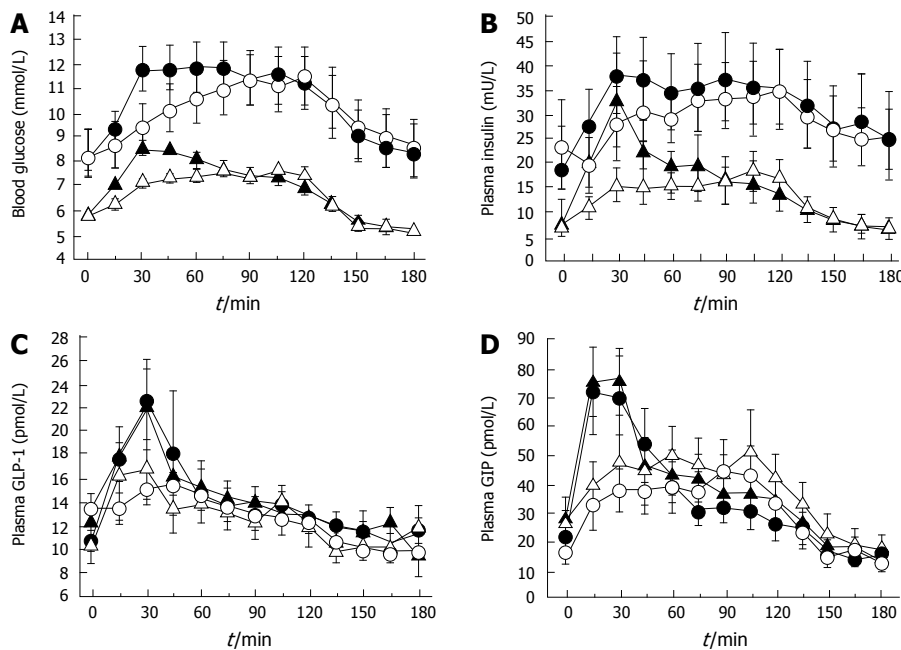


Figure 3 Effect of initially more rapid intraduodenal glucose infusion (3 kcal/min between $t = 0$ and 15 min and 0.71 kcal/min between $t = 15$ and 120 min) (closed symbols) compared to constant infusion (1 kcal/min between $t = 0$ and 120 min) (open symbols) in healthy subjects (triangles) and patients with type 2 diabetes (circles) on (A) blood glucose, (B) plasma insulin, (C) plasma GLP-1, and (D) plasma GIP. Each pair of curves differs between 0 and 30 min for variable vs constant intraduodenal infusion ($P < 0.05$). Adapted from O'Donovan *et al* 2004^[52].

subjects and patients with type 1 diabetes^[67]. Hence, the effect of transient hyperglycemia on small intestinal glucose absorption remains uncertain.

Given that an upper limit exists for absorption of glucose across the small intestinal mucosa, it is logical that patterns of intestinal motility which serve to spread luminal glucose over a large surface area could promote glucose absorption. Furthermore, certain motor patterns could facilitate mixing of complex carbohydrates with digestive enzymes, and their exposure to brush border disaccharidases. Thus, when glucose is infused directly into the duodenum, its rate of absorption increases with the number of duodenal pressure waves and propagated pressure wave sequences^[54,67]. Further insights into the effects of luminal flow on glucose absorption are likely to require additional techniques, such as intraluminal impedance measurement, in which inferences can be made regarding movement of fluid boluses by measuring changes in electrical impedance between sequential pairs of intraluminal electrodes. A preliminary study in healthy humans using this technique indicates that pharmacological suppression of intraduodenal flow with an anticholinergic agent is associated with delayed absorption of luminal glucose^[68]. These observations are likely to be of relevance to patients with type 1 diabetes mellitus, who demonstrate an increased frequency of small intestinal pressure waves in the postprandial state^[67].

The region of small intestine that is exposed to carbohydrate is also likely to be a determinant of the glycemic response. GLP-1 is released from intestinal L cells, whose concentration is greatest in the distal jejunum, with fewer L cells located in the proximal jejunum, ileum, and colon^[69]. In humans, it is unclear whether nutrients must interact directly with L cells to stimulate GLP-1 release. A neural or endocrine loop between the duodenum and the more distal small bowel has been postulated^[70], but remains unproven. The lack of a GLP-1 response when glucose is infused into the duodenum below a certain dose (1.4 kcal/min)^[71] would be consistent with the concept of complete absorption of the glucose load high

in the small intestine, precluding significant interaction with L cells, although other investigators have reported a GLP-1 response even with 1 kcal/min intraduodenal glucose infusion^[52]. Nevertheless, GLP-1 responses to meals are enhanced following intestinal bypass procedures that promote access of nutrients to more distal small intestine^[72-75], while inhibition of sucrose digestion in the proximal small intestine with acarbose increases the GLP-1 response^[76], presumably by facilitating more distal interaction of the intestine with glucose. It follows that dietary modifications that favor exposure of more distal small intestinal segments to glucose could reduce glycemic excursions by stimulating GLP-1 release. Furthermore, a major action of both GLP-1 and peptide YY, which is also released from L cells, is to retard gastric emptying, thus slowing any further entry of carbohydrate to the small intestine^[77].

IMPACT OF VARIATIONS IN GLYCEMIA ON UPPER GUT MOTILITY

Acute changes in the blood glucose concentration are now recognised to have a major, reversible impact on the motor function of every region of the gastrointestinal tract. This may in part account for the poor correlation of upper gastrointestinal dysfunction in diabetes with evidence of irreversible autonomic neuropathy, to which it has traditionally been attributed^[78]. When compared to euglycemia (4-6 mmol/L), gut motility is modulated through the range of blood glucose concentrations from marked hyperglycemia (greater than 12 mmol/L), through "physiological" blood glucose elevation (8-10 mmol/L), to insulin-induced hypoglycemia (less than 2.5 mmol/L), and effects are observed rapidly (within minutes), although the thresholds of response may differ between gut regions^[6]. The mechanisms mediating the effects of acute changes in the blood glucose concentration are poorly defined, and the potential impact of chronic, as opposed to acute, variations in glycemia on gastrointestinal motility has hitherto received little attention. Nevertheless, it is clear

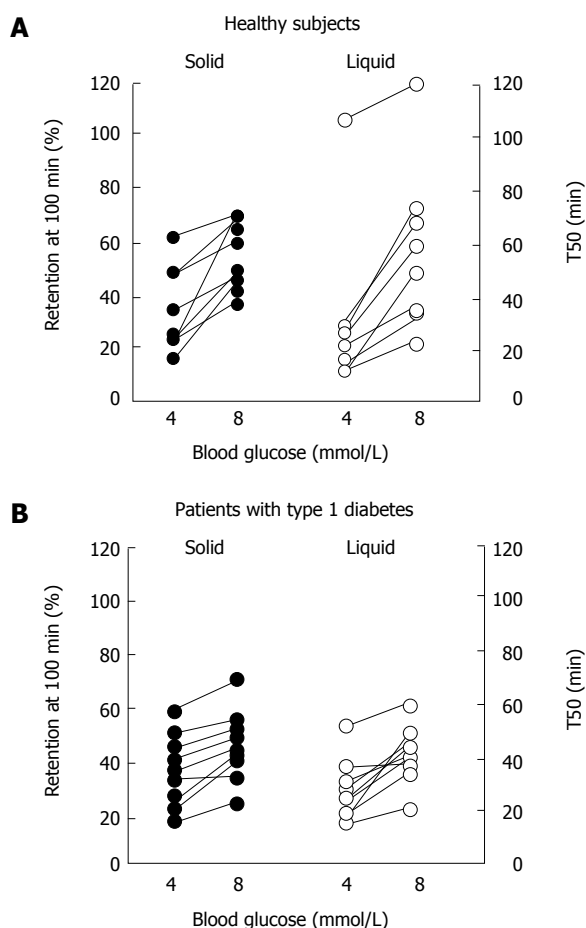


Figure 4 Solid and liquid gastric emptying in (A) healthy subjects and (B) patients with type 1 diabetes mellitus during euglycemia (blood glucose 4 mmol/L) and "physiological" hyperglycemia (blood glucose 8 mmol/L). Adapted from Schvarcz *et al* 1997^[82].

that gut motor function and postprandial glycemia are highly interdependent variables.

Gastric motility

Marked hyperglycemia (16–20 mmol/L) slows both solid and nutrient liquid emptying in patients with type 1 diabetes when compared to euglycemia (5–8 mmol/L)^[79]; in type 2 patients, cross-sectional data also indicate an inverse relationship between the blood glucose concentration and the rate of gastric emptying^[49]. Conversely, gastric emptying is accelerated by acute hypoglycemia induced by insulin (2.6 mmol/L) in healthy subjects^[80] and type 1 patients, even when emptying is slower than normal during euglycemia^[81]. In type 1 diabetes, as well as healthy volunteers, elevation of blood glucose to "physiological" postprandial levels (8 mmol/L) also slows gastric emptying when compared to euglycemia (4 mmol/L)^[82] (Figure 4). The magnitude of the effects of glycemia on the rate of gastric emptying is substantial, and has implications for absorption of orally administered medications, including oral hypoglycemic agents^[83], as well as impacting on carbohydrate absorption.

The rate of gastric emptying is determined by the coordinated activity of various regions of the stomach and proximal small intestine^[84]. The proximal stomach acts as a reservoir for solids and generates tonic pressure to facilitate liquid emptying. The distal stomach grinds

and sieves solids and pumps chyme across the pylorus, predominantly in a pulsatile manner, while phasic and tonic pyloric pressures, and duodenal contractile activity act as a brake to gastric outflow. The timing of antral contractions is controlled by an electrical slow wave, with a frequency of about 3 per minute, generated by the interstitial cells of Cajal^[85]. During fasting, cyclical activity of gastric motility is observed, with a periodicity of about 90 min, characterised by irregular contractions of increasing frequency (phase II), and a brief (5–10 min) period of regular contractions at the rate of 3 per minute (phase III) during which indigestible solids empty from the stomach, followed by motor quiescence (phase I). Acute hyperglycemia is associated with diminished proximal gastric tone^[86–88], suppression of both the frequency and propagation of antral pressure waves^[89–92], and stimulation of pyloric contractions^[93]—a motor pattern associated with slowing of gastric emptying. The frequency of the gastric slow wave is also disturbed^[94]. The suppression of antral motility is observed at blood glucose concentrations as low as 8 mmol/L^[89,91]; the threshold appears higher in the proximal stomach^[95]. Hyperglycemia also attenuates the prokinetic effects of erythromycin in both healthy subjects and type 1 patients^[96,97], at least in part by inhibiting the stimulation of antral waves and coordinated antroduodenal pressure sequences^[98]. This effect is of considerable importance, since the action of other prokinetic drugs is also likely to be impaired during hyperglycemia, although this issue has not been specifically examined.

Small intestinal motility

As in the stomach, fasting small intestinal motility is cyclical, and characterised by phases I to III, the latter with a frequency of 9 to 12 per minute. This "migrating motor complex" (MMC) propagates aborally along the small intestine, and serves to "sweep" the lumen of indigestible debris. After a meal, the MMC is interrupted by irregular contractions propagated over short distances, which facilitate digestion and absorption of nutrients.

In healthy subjects during hyperglycemia (10 mmol/L), the duodenum becomes less compliant (more "stiff") to balloon distension, while distension stimulates a greater number of phasic pressure waves, when compared to euglycemia^[99]. Both phenomena could contribute to a duodenal "brake" to gastric emptying. More marked hyperglycemia (12–15 mmol/L) reduces the cycle length of the MMC^[100], the frequency of duodenal and jejunal pressure waves, and the duration of the postprandial period (early return of phase III activity), and slows small intestinal transit^[101]. These alterations in function could have implications for absorption of nutrients and medications, alteration in bowel habit, and the occurrence of small intestinal bacterial overgrowth in diabetes^[102]. However, other than suppression of proximal duodenal wave frequency^[103], there is limited information about the effects of hyperglycemia on small intestinal motor function in patients, as opposed to healthy volunteers.

Mechanisms mediating the effects of hyperglycemia on gastric and small intestinal motility

Most information about the etiology of gastrointestinal dysfunction in diabetes relates to the effects of

longstanding diabetes rather than acute, reversible changes that could relate to transient hyperglycemia^[104]. Rodent models of diabetes have demonstrated marked apoptosis of enteric neurons^[105], affecting nitrergic (inhibitory) neurons in particular^[106], and loss of interstitial cells of Cajal^[107]. The latter are also deficient in humans with diabetes and severe gut symptoms^[108]. Hyperglycemia appears to be responsible for apoptosis of enteric neurons^[109], but the latter would seem unlikely to mediate changes that are evident within minutes, rather than days or weeks. Enteric neurons sensitive to changes in glucose have been identified^[110], although their responsiveness to systemic, as opposed to luminal glucose remains unclear. Vagal nerve function is reversibly inhibited by acute hyperglycemia^[111,112], and this may account for some of the observed phenomena. Hyperinsulinemia is unlikely to explain the observed effects, particularly as they are seen in type 1 (insulin deficient) as well as type 2 and healthy subjects. Studies are indicated to determine whether reversible changes in nitrergic or serotonergic neurotransmission occur with variations in glycemia.

THERAPEUTIC STRATEGIES DIRECTED AT MINIMISING POSTPRANDIAL GLYCEMIA

The major impact of gastrointestinal function on the glycemic response to meals, as outlined above, suggests a number of logical, and in many cases complimentary, strategies to lower postprandial blood glucose concentrations (Table 1). These include (1) minimising the carbohydrate content, or substituting low- for high-glycemic index foods in meals, (2) slowing gastric emptying, even in individuals with modest delays in emptying provided they remain free of symptoms, (3) inhibiting the absorption of carbohydrate from the small intestine, or delaying its absorption to more distal small intestinal segments, and (4) augmenting the incretin response. Many approaches fulfill a number of these aims concurrently. Most studies relating elevated postprandial glycemia with cardiovascular risk have evaluated blood glucose 2 h after a meal^[113], suggesting that lowering peak blood glucose may be an appropriate target. Nevertheless, the glycated hemoglobin relates closely to the integrated mean blood glucose (i.e. area under the curve) over 24 h, albeit with a curvilinear rather than linear relationship^[114], so reducing the total area under the blood glucose excursion over several hours after a meal may also be an important goal. It should be noted that strategies for individuals with impaired glucose tolerance or type 2 diabetes managed without exogenous insulin, particularly those involving slowing of gastric emptying, may not be applicable to type 1 and insulin-requiring type 2 patients, in whom the goal should be to coordinate the absorption of carbohydrate with the action of exogenous insulin, which in some cases may involve accelerating gastric emptying, if it is already delayed^[115].

Carbohydrate content and glycemic index

Low-carbohydrate diets were the mainstay of treatment for diabetes in the pre-insulin era^[116]. The outcome of the Nurses' Health Study is indicative of a relationship between both cardiovascular risk and the incidence of diabetes with dietary glycemic load^[117]. Short-term

Table 1 Therapeutic strategies directed at minimizing postprandial glycemia

Strategy	Examples
Alter carbohydrate in diet	Low-carbohydrate diets Low glycemic index carbohydrates
Slow gastric emptying	High fiber diets Fat "preloads" Low glycemic index carbohydrates Acarbose GLP-1 analogs and DPP-IV inhibitors Pramlintide
Inhibit small intestinal carbohydrate absorption	Acarbose High fiber diets Low glycemic index carbohydrates
Augment the incretin response	GLP-1 analogs and DPP-IV inhibitors Acarbose Fat "preloads" ? Low glycemic index carbohydrates

studies indicate the potential for low-carbohydrate diets to improve 24 h glycemia and glycated hemoglobin in patients with type 2 diabetes^[118], including those failing conventional treatment with diet and a sulfonylurea^[119]. In medium- to long-term studies, substituting protein for carbohydrate improved glycemia in overweight hyperinsulinemic subjects^[120], while a low-carbohydrate diet improved fasting glucose over 6 mo in type 2 patients, with glycemic benefits maintained at 1 year, when compared to a low-fat diet^[121,122]. The magnitude of the decrease in glycated hemoglobin was small (mean 0.6% in the latter study), but likely to be clinically significant. In addition to the reduction in carbohydrate load, protein itself might improve glycemia by stimulating insulin release^[123], although this phenomenon is less apparent in medium-versus short-term studies^[124].

Rather than trading carbohydrates for alternative macronutrients, another approach is to substitute low- for high-glycemic index carbohydrates. The glycemic index (GI) compares the blood glucose response of a test food with that of a standard carbohydrate, either glucose or white bread^[125]. Foods may be low GI by virtue of a relative delay in gastric emptying and/or small intestinal glucose absorption^[126,127]. For example, spaghetti (low GI) empties from the stomach much slower than potato (high GI) from about 60 min after the meal, although their glycemic profiles diverge earlier^[128], indicating that slowing of small intestinal glucose absorption is important. Both the physical properties of the carbohydrate (such as enclosed kernels) and its chemical composition (such as a high amylose:amylopectin ratio) influence small intestinal carbohydrate digestion and absorption^[127,129]. Glycemic index tends to vary inversely with the content of dietary fiber in meals^[130]; dietary fiber *per se* potentially slows gastric emptying^[131] and small intestinal carbohydrate absorption^[132], the latter by a mechanism that includes modification of small intestinal motility from a stationary (favoring mixing), to a propulsive pattern. The beneficial effect on the glycemic response of adding guar gum to an oral glucose load appears to be achieved mainly by slowing gastric emptying^[133,134]. Nevertheless, guar also slows

small intestinal glucose absorption, probably by inhibiting diffusion of glucose out of the luminal contents^[135]; this is reflected in the observation that both GLP-1 and insulin responses are less than with guar-free glucose^[136].

Low GI foods may also stimulate insulin release through the incretin effect, or other mechanisms^[137]. Furthermore, they may enhance satiety and reduce energy intake at a subsequent meal^[138,139]. Additional information about the potential for these beneficial effects from different classes of low GI foods is needed. Fructose has been advocated as a low GI substitute for glucose in the diabetic diet, since it results in a much lower glycemic excursion than an equivalent glucose load^[140], while retaining the capacity to stimulate insulin secretion^[141]. In addition, some investigators have found that fructose ingestion suppresses food intake more than glucose^[140,142], although this issue is controversial^[141], and probably depends on the load and timing of fructose ingestion in relation to the subsequent meal.

Most medium- to long-term studies of low GI diets indicate a benefit for glycemic control^[143], typically the effect is modest^[144], but is still likely to be clinically meaningful, and in many cases is of similar magnitude to the improvement in glycemic control achieved by pharmacological agents^[145].

Slowing gastric emptying

Given the relationship between the degree of postprandial glycemia and the rate of gastric emptying in both healthy subjects^[27] and type 2 patients^[28], it is logical that dietary and/or pharmacological interventions which slow gastric emptying should be effective in lowering postprandial glycemia in type 2 diabetes. In addition to the effects of dietary fiber in retarding gastric emptying, slowing of emptying by either an oral proteinase inhibitor^[146], adding a solid non-carbohydrate meal to an oral glucose load^[147], or combining fat (the most potent macronutrient for slowing gastric emptying^[148]) with carbohydrate^[149], all reduce postprandial blood glucose and insulin responses. The underlying concept is that the presence of nutrients in the small intestine both delays gastric emptying and stimulates GLP-1 and GIP. Hence, consumption of oil as a “preload” before a mashed potato meal markedly delays the subsequent rise in blood glucose, and stimulates GLP-1 release in patients with type 2 diabetes^[150]. This approach requires further refinement to determine the optimum load, timing, and macronutrient content of the “preload”, but has the potential advantage of simplicity when compared to pharmacological strategies, which also appear to act predominantly by slowing gastric emptying. For example, the improvement in postprandial glycemia associated with GLP-1 and its analogs is related to slowing of gastric emptying rather than enhancement of insulin secretion^[40]; the latter is in fact reduced due to a decrease in the rate of entry of carbohydrate into the small intestine. The amylin analog, pramlintide, also slows gastric emptying^[151], and its use is associated with an improvement in overall glycemic control, as assessed by glycated hemoglobin, with medium-term use in type 1 and type 2 patients^[152-155]. Pramlintide has the additional advantage of promoting weight loss, probably by suppressing appetite^[156].

Inhibiting absorption of glucose

The α -glucosidase inhibitors, including acarbose, delay absorption of carbohydrates in the proximal small intestine^[157]. The resultant exposure of more distal intestinal segments to glucose results in enhanced and prolonged GLP-1 secretion in healthy subjects^[76,158], with subsequent slowing of gastric emptying^[159]. The magnitude of these affects is likely to depend on meal content (ie. disaccharide load). Moreover, acarbose fails to stimulate GLP-1 release or delay gastric emptying in patients with type 2 diabetes, although it is still beneficial for reducing postprandial glycemia in this group^[160], presumably by impairing carbohydrate absorption. Inhibition of glucose entry into enterocytes might represent an additional mode of action of acarbose^[161]. Again, it is clear that the mechanisms by which postprandial glycemia is improved frequently overlap. For example, slowed absorption of glucose, as discussed, is also a feature of low GI and high fiber diets.

Augmenting the incretin response

The effect on GLP-1 concentrations of the dietary strategies already discussed, and the observed potentiation of GLP-1 secretion and associated improvement in glycemic control after bariatric surgery^[162], point to the value of augmenting the incretin response in optimising postprandial glycemia. GLP-1 is metabolised rapidly by the enzyme dipeptidyl peptidase IV (DPP-IV), and therefore is not a suitable agent for therapeutic administration. Instead, longer lasting agonists have been developed, including both albumin-bound analogs of GLP-1, and exendin-4, a peptide derived from the saliva of the Gila monster lizard, which is structurally similar to GLP-1 and shares several biological properties, but may be a more potent insulinotropic agent than GLP-1^[26]. Subcutaneous administration has been shown to reduce postprandial glycemia in type 2 patients^[163]. Resistant analogs of GLP-1, along with DPP-IV inhibitors, appear to have a promising role in the therapy of diabetes^[164].

CONCLUSION

Upper gastrointestinal function plays a major, though often overlooked, role in determining postprandial glycemia. Recent insights into the mechanisms by which variations in gut function alter the blood glucose concentration have suggested a number of potential therapeutic strategies that require further evaluation for their utility in achieving good glycemic control.

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REVIEW

Assessing risks for gastric cancer: New tools for pathologists

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Abstract

Although the Sydney Systems (original and updated) for the classification of gastritis have contributed substantially to the uniformity of the reporting of gastric conditions, they lack immediacy in conveying to the user information about gastric cancer risk. In this review, we summarize the current understanding of the gastric lesions associated with an increased risk for cancer, and present the rationale for a proposal for new ways of reporting gastritis. In addition to the traditional histopathological data gathered and evaluated according to the Sydney System rules, pathologists could add an assessment expressed as grading and staging of the gastric inflammatory and atrophic lesions and integrate these findings with pertinent laboratory information on pepsinogens and gastrin levels. Such an integrated report could facilitate clinicians' approach to the management of patients with gastric conditions.

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INTRODUCTION

Chronic gastritis is an inflammatory condition of the gastric mucosa characterized by elementary lesions whose type, extent, and distribution are related to their etiology and modulated by host responses and environmental factors^[1]. Infection with *H pylori*, which affects an estimated three to four billion persons worldwide, is by far the most

common cause of chronic active gastritis; chemical agents, autoimmune phenomena, and other infections account for a very small proportion of chronic, usually non-active gastritides. *H pylori*-gastritis is epidemiologically and biologically linked to the development of gastric cancer^[2] and *H pylori* has been listed as a class I carcinogen^[3]. Epidemiological and pathological data suggest that extent, intensity, and distribution patterns of gastric inflammation and atrophy are consistently related to the incidence of gastric cancer in a population^[4-7]. Although odd-ratios for gastric cancer and peptic ulcer risk in relationship with the type of gastritis have been estimated, most often retrospectively, only in small series and in few populations^[5,8-10], it is widely accepted that the accurate histopathological assessment of the gastric mucosa could serve as a reasonably good predictor of cancer risk in an individual patient. In fact, most recent classifications of gastritis have contained the implicit aim of providing a clinico-pathological correlation that could be both synchronous (that is, at the time of the sampling) and, more usefully, diachronic.

When appropriate sampling is available, the histopathological features of the gastric mucosa recognized as being part of the neoplastic process and broadly referred to as "pre-neoplastic lesions" (atrophy, pyloric and intestinal metaplasia, epithelial dysplasia) can be accurately evaluated by the microscopic examination of mucosal biopsies. Although classification systems such as the Sydney System^[11], its Houston-updated version^[12], and the more recent guidelines for the evaluation of atrophy^[13] suggest strategies for the formulation of histopathological reports, we still lack a way to translate the pathological information into a standardized report that would convey comprehensive information on the gastric condition while lending itself to a straightforward analysis of cancer risk.

The purpose of this article is to explore ways for pathologists to maximize the predictive value of the gastric evaluation by: (1) streamlining the histopathological report of gastric biopsies, and (2) integrating relevant laboratory information with pathological data.

GASTRIC MUCOSAL CHANGES RELATED TO GASTRIC CANCER

As a result of seminal field studies conducted by Max Siurala in Finland and Estonia^[14-17] and Pelayo Correa in Colombia^[4,18-19], as well as the crucial body of knowledge derived from decades of Japanese studies^[20,21], the separate entities of chronic superficial gastritis, atrophy, metaplasia, dysplasia and carcinoma were integrated into a hypothetical

sequence known as Correa's cascade^[22]. Increasingly well-documented by patho-epidemiological studies, the 1984 multi-step hypothesis of gastric carcinogenesis still lacked an etiological initiator. The missing first step was discovered in the same year^[23] and *H pylori* found its place at the top of the cascade^[24].

The histopathological lesions broadly regarded as preneoplastic are chronic gastritis, atrophy, intestinal metaplasia, dysplasia, and neoplasia. Their evolution in a cohort can be viewed as a pyramid with a very large base representing the entire *H pylori*-infected population; a segment of these subjects (generally larger in developing than in industrialized areas) will progress to atrophic gastritis, mostly accompanied by intestinal metaplasia. A very small minority will progress further to dysplasia with some eventually developing adenocarcinoma. The closer a lesion is to neoplasia, the more likely it will progress into it. Thus, whereas chronic gastritis is a remote and uncertain precursor of gastric cancer that could be better called a "predisposing condition," high-grade dysplasia is considered already a neoplastic lesion^[25,26]. If pathologists could make a reliable assessment of the risk that each patient has, based on a staging of the disease, effective strategies could be developed to detect the early, curable phase of gastric cancer and prevent its progression.

Chronic gastritis

The risk of gastric cancer for a patient with simple, non-atrophic *H pylori* gastritis is negligible, thus, the decision to treat the infection is based, in most cases, on other considerations. There is, however, one exception. Gastric cancer and atrophic gastritis associated with it have at least some familial predisposition^[27-30]; therefore, it would seem wise to treat *H pylori* infection as early as possible in direct relatives of patients with gastric cancer. This is one of the rare circumstances in which *H pylori* would be eradicated for the specific purpose of preventing gastric carcinoma in an individual patient.

Atrophy

Gastric atrophy is defined as the loss of appropriate glands in a given gastric compartment^[13,31]. This purely histopathological definition indicates that the glands expected to be present in the portion of gastric mucosa under examination (for example, oxyntic glands in the mucosa of the corpus) are no longer there, and have been replaced by something else that does not belong to that area. This "something else" may be extracellular matrix, fibroblasts and collagen, or other glands that normally are not there (e.g., intestinal-type glands or pseudo-pyloric glands). Any of these replacements prevents that portion of gastric mucosa from performing its normal functions (e.g., to secrete acid). Thus, the functional correlate of atrophy is strictly related to its extension.

Atrophic gastritis is a condition characterized by the presence of significant areas of atrophy. Its two most common underlying causes are chronic infection with *H pylori* and the autoimmune gastritis that may become associated with pernicious anemia. In the Updated Sydney System, the term "atrophic gastritis" is used in contrast to

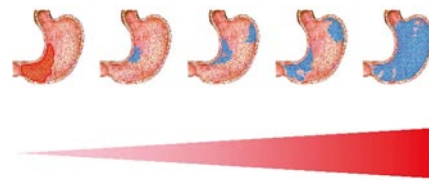


Figure 1 Schematic representation of the progression of atrophy, from absent in the case of antrum-predominant non-atrophic gastritis depicted on the left to the almost generalized metaplastic atrophy depicted on the right. The increased extension of atrophy corresponds to an increased cancer risk, indicated as an expanding triangle. The extension of atrophy can also be reported as a stage from 0 to 4.

"non-atrophic gastritis" or simply "gastritis," a condition usually more severe in the antrum (hence the term "antral-predominant") found in most subjects infected with *H pylori* in the Western industrialized world.

The stomach affected by atrophic gastritis shows a decrease or absence of appropriate glands, an expansion of the antral-type mucosa into the corpus ("antralization" or pseudo-pyloric metaplasia) and usually extensive areas of intestinal metaplasia. This condition has been known for several decades to represent a significant epidemiological risk factor for gastric adenocarcinoma^[14,17,24,32-36]; as schematically depicted in Figure 1, its prognostic implications in the individual patient seem to be related to the extent and distribution of the atrophic areas^[10,37].

Intestinal metaplasia

Intestinal metaplasia is the replacement of the normal gastric mucosa with an epithelium similar to that of the intestine. Attempts to classify the different types of intestinal metaplasia have resulted in a confusing terminology (complete *vs* incomplete, type 1, 2a and 2b, *etc.*); the classification currently used was proposed by Jass and Filipe^[38,39]: Type I (brush border and no sulfomucins); Type II (no brush border, rare sulfomucins); and Type III (no brush border, cellular disarray, abundant sulfomucins). Type I intestinal metaplasia has been often said to pose little increased risk of developing carcinoma, whereas type III has been considered as an already dysplastic lesion^[10,40-42]. The classification of the three types of metaplasia requires relatively sophisticated histochemical techniques and is far from being standardized. Furthermore, the data suggesting different cancer risks for the different types of intestinal metaplasia are not unequivocal^[43]. Therefore, immunohistochemical sub-typing of intestinal metaplasia should be limited to the clinical research setting and not a part of the routine evaluation of patients with intestinal metaplasia.

Dysplasia

Malignancy is the final step of progressive genetic and phenotypic changes that modify the original cellular morphology, eventually generating a biologically new cell characterized by uncontrolled growth and the potential to migrate and implant in locations beyond its original fixed

site. This biological process has been called multi-step or step-wise oncogenesis. In epithelial tissues (for example, the squamous lining of the uterine cervix or the columnar lining of the colon) the first of step visible to an observer using a light microscope is a change in the morphology of the cells that form the epithelium. Nuclei are larger, nucleoli may be prominent and the chromatin may be clumped or granular; compared to the larger nucleus, the cytoplasm appear smaller, a phenomenon referred to as “increased nucleo-cytoplasmic ratio”. Various degrees of disarray of the orderly structure of the normal epithelium usually accompanying these changes. Epithelial alterations of this kind occur in two situations: when the epithelium has been injured and is undergoing repair, and when genetic alterations have transformed the cells in a neoplastic growth. It is generally agreed by pathologists that in the former instance one refers to the phenomenon as “regenerative atypia”, whereas in the latter case the term “dysplasia” is used^[44].

The importance of recognizing and correctly identifying dysplasia is self-evident: while regenerative atypia is the desired response to epithelial injury and an essential part of an organism's homeostasis, dysplasia is the harbinger of cancer and requires immediate action. However, the morphological differences between atypia and dysplasia are not always apparent, and significant areas of phenotypic overlap exist between the two. Pathologists have tried for years to standardize the criteria for the diagnosis and grading of dysplasia in tissues accessible to biopsy sampling. Without getting into the complex historical details of the process, for the purpose of this review we say only that, through the efforts of pioneers such as the late Rodger Haggitt, Robert Riddell, Brian Reid, and others, a satisfactory level of agreement has been reached for dysplasia of the colon and of Barrett's epithelium^[44-46]. Gastric dysplasia has received less attention in the past, with only one major consensus article addressing the issue before 1996^[47].

In the last decade, the discovery of *H pylori* and its relationship with gastric cancer has stimulated increasing attention to the preneoplastic lesions of the stomach. The possibility that curing this infection could prevent or even cause the regression of such lesions has highlighted the need for uniform and rigorous definitions and diagnostic criteria. However, unlike metaplasia, whose recognition has always been largely free of major disputes, or atrophy, which has been the focus of major conceptual disagreements among pathologists, dysplasia exposed a novel angle of controversy: a pathological schism between East and West, or, more accurately, between Japan and the West^[48].

Japan is one of the countries with the highest incidence of adenocarcinoma of the stomach in the world; at the same time, it also has the world's best survival rates for gastric cancer. Although the effective early detection programs, innovative endoscopic techniques, and daring and successful therapeutic endoscopists have been invoked to explain the Japanese success in this area, another explanation has been suggested, mostly in a veiled or oblique manner. To state it simply, it has been implied that, to have such good survival rates the Japanese must

call cancer what others call dysplasia. The question has been propelled into the international scientific forum only recently, through the efforts of RJ Schlemper, who in 1996 organized a workshop to address the issue. This workshop resulted in a seminal paper entitled “Differences in diagnostic criteria for gastric carcinoma between Japanese and Western pathologists,” published in the Lancet in 1997^[49]. Following the workshop and publication of its findings, several other groups have formed to tackle the problem in the traditional pathologists' fashion: by trying to measure the level of agreement (or disagreement) amongst observers. These groups included various proportions of Japanese and Western pathologists, and the ultimate aim was to reach a consensus that classification, if used globally, would allow comparative studies performed in different countries. As a result, new issues have emerged and new classifications have been proposed. The classification currently accepted by the World Health Organization^[50] is largely modeled on the consensus agreement reached in Padua, Italy, in 1998^[26], and summarizes one of the most recent proposals for an integrated therapeutic and pathological approach^[51].

The Padova model is based: (1) on the definition of dysplasia as pre-invasive neoplasia; and (2) on a five-category classification of gastric neoplasia which includes: 1, negative for dysplasia; 2, indefinite for dysplasia; 3, non-invasive neoplasia; 4, suspicious for invasive cancer; 5, gastric cancer. The numerical prefix assigned to each diagnostic category essentially corresponds to the diagnostic categories of the Japanese Classification for Gastric Cancer^[52]. Within each category one or more sub-categories are hierarchically ordered to cover the spectrum of epithelial alterations.

THE IMPORTANCE OF GOOD SAMPLING

The topographic distribution of inflammatory infiltrates, lymphoid follicles, atrophy, and metaplasia is an essential determinant used for all classifications of gastritis. These changes may be patchily distributed and their relative intensity in different parts of the stomach may be highly variable. Furthermore, the inflammatory and atrophic processes have different phenotypical expressions in different regions of the stomach. Therefore, to obtain an accurate picture of gastritis, pathologists must have a set of specimens representative of each gastric compartment. Each specimen is examined according to uniform criteria, a general impression of the intensity of the features of gastritis is extrapolated from the various specimens from each compartment, and finally this information is amalgamated in a topographical diagnosis. The location of the biopsy specimens recommended by the Updated Sydney System^[12] is depicted in the left panel of Figure 2. A suggestion has been made to replace the original sites with others, purportedly more likely to yield information about the extension of intestinal metaplasia^[53], but in the absence of independent testing no proposal in this sense has been presented.

Irrespective of the protocol used, gastroenterologists must keep in mind that the predictive information they can get from their pathologist is only as good as the

biopsy sampling submitted for examination. The Sydney System 5-biopsy protocol is a compromise between what is practically doable in routine practice and the ideal need for maximal topographic information. As depicted in Figure 2, right panel, in special situations such as the diagnosis and follow-up of gastric mucosa-associated lymphoid tissue B-cell lymphomas or the diachronic investigation of dysplasia much more extensive sampling protocols need to be applied^[48,54-56].

VIRTUAL HISTOPATHOLOGY

The determination of serum pepsinogens I (PG I) and II (PG II), gastrin-17 (G-17) and IgG anti-*H. pylori* antibodies by ELISA has been proposed as an array of non-invasive markers for the assessment of both morphological and functional status of the gastric mucosa^[57]. The rationale for this approach, described by its enthusiastic supporters as the “serological biopsy,” rests on the fact that PG I is exclusively secreted by oxyntic glands and represents an excellent marker of the secretory ability of the gastric corpus. In contrast, PG II is produced by all types of gastric and duodenal glands and its production is influenced by gastric inflammation^[58,59]. Although these molecules are secreted into the gastric lumen, small amounts seep out into the bloodstream and can be measured. Gastrin-17 (G-17), produced in the antrum and secreted directly into the blood, is a specific marker of G cell function^[60]. Several studies have now shown that serum levels of PG I, PG II and G-17 are high in subjects with *H. pylori* non-atrophic chronic gastritis^[61]. Both PG I and PG II concentrations are found to decrease significantly two months after the eradication of *H. pylori*^[62,63]. Furthermore, the ratio of PG I and G-17 levels have been found to correlate well with the histopathological diagnosis of atrophic body gastritis and, in some studies, to be associated with the presence of gastric cancer^[64-66].

In a recent study, De Mario and his colleagues^[67] demonstrated that the analysis of serum pepsinogens, G-17 and anti-*H. pylori* IgG levels provide consistent and reproducible information regarding gastric atrophy and its association with *H. pylori*. The authors suggest that dyspeptic patients with normal PG I, PG II, G-17 and a negative serological test for *H. pylori* can be reassured that they are unlikely to have peptic ulcer disease and can be treated symptomatically. In contrast, patients with panel test results indicating *H. pylori*-related chronic gastritis, with or without atrophy, could either be treated for *H. pylori* or referred for endoscopy, depending on the type and severity of their manifestations.

GENERATING A CLINICALLY USEFUL HISTOPATHOLOGY REPORT

The article reporting the Updated Sydney System, published in October 1996, has recently passed the 1000-citation milestone^[68], suggesting that the semi-quantitative scoring system it advocated remains a useful tool for clinical research. Nevertheless, the same pathologists who use it when assessing biopsies for clinical studies find it too cumbersome to use in their routine

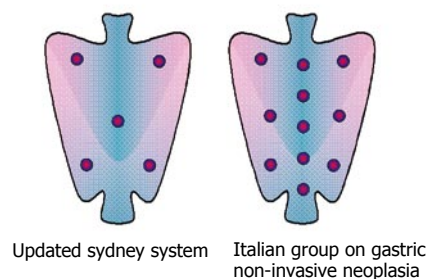


Figure 2 Two different biopsy protocols.

diagnostic activities.

Using the framework provided by the Sydney System's and the Atrophy Club's analytic approach, we have recently put forward a proposal for a grading and staging scheme that integrates the relevant histopathological data gathered and interpreted by the pathologist and delivers them in the form of a simple, yet information-rich report^[69]. We have suggested that the method is both feasible and practical, and that staging and grading (preceded by a description of the histological findings in the biopsy samples) could represent the concluding message of the histological report. This scheme could be done for chronic gastritis what the grading and staging system introduced by the International Group of Hepatologists in 1995 did for chronic hepatitis: make prognostically significant and reproducible information immediately available in the clinical practice^[70,71].

Briefly, the proposal consists of summarizing the combined intensity of mononuclear and scoring granulocytic inflammation in both antral and oxyntic biopsy samples in a grade from 0 (no inflammation) to 4 (a very dense infiltrate in all the biopsy samples). The extent of atrophy, with or without intestinal metaplasia, would be reported as a stage from 0 (no atrophy) to 4 (pan-atrophy involving all antral and oxyntic samples). The latter would convey information on the anatomical extent of the atrophic-metaplastic changes related to cancer risk. Figure 1 shows the progression from stage 0 (left) to stage 4 (right).

A pathologist who had access to the results of the “serological biopsy” and applied the grading and staging principles outlined in this scheme could generate a comprehensive informative integrated report that could be used by clinicians as a solid base for the management of patients with gastric conditions.

This proposal has been discussed at an international consensus group of gastroenterologists and pathologists (Operative Link for Gastritis Assessment-OLGA) that gathered in Parma, Italy, in April 2005. The group included Massimo Rugge, Padova, Italy; Pelayo Correa, New Orleans, Louisiana, USA; Francesco Di Mario, Parma, Italy; Emad El-Omar, Aberdeen, Scotland, UK; Roberto Fiocca, Genova, Italy; Karel Geboes, Leuven, Belgium; David Y Graham, Houston, Texas, USA; Takanori Hattori, Shiga, Japan; Peter Malfertheiner, Magdeburg, Germany; Pentti Sipponen, Espoo, Finland; Joseph Sung, Hong Kong, China; Wilfred Weinstein, Los Angeles, California,

USA; Michael Vieth, Bayreuth, Germany; and Robert M Genta, Geneva, Switzerland.

After deliberations that led to a number of modifications of the original proposal, the OLGA group has agreed that an international staging method is needed to advance research in gastritis and is preparing to test its feasibility and reproducibility both in retrospective and prospective multi-center studies.

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GASTRIC CANCER

Resveratrol engages selective apoptotic signals in gastric adenocarcinoma cells

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Abstract

AIM: To investigate the intracellular apoptotic signals engaged by resveratrol in three gastric adenocarcinoma cancer cell lines, two of which (AGS and SNU-1) express p53 and one (KATO-III) with deleted p53.

METHODS: Nuclear fragmentation was used to quantify apoptotic cells; caspase activity was determined by photometric detection of cleaved substrates; formation of oxidized cytochrome C was used to measure cytochrome C activity, and Western blot analysis was used to determine protein expression.

RESULTS: Gastric cancer cells, irrespective of their p53 status, responded to resveratrol with fragmentation of DNA and cleavage of nuclear lamins A and B and PARP. Resveratrol, however, has no effect on mitochondria-associated apoptotic proteins Bcl-2, Bcl-xl, Bax, Bid or Smac/Diablo, and did not promote subcellular redistribution of cytochrome C, indicating that resveratrol-induced apoptosis of gastric carcinoma cells does not require breakdown of mitochondrial membrane integrity. Resveratrol up-regulated p53 protein in SNU-1 and AGS cells but there was a difference in response of intracellular apoptotic signals between these cell lines. SNU-1 cells responded to resveratrol treatment with down-regulation of survivin, whereas in AGS and KATO-III cells resveratrol stimulated caspase 3 and cytochrome C oxidase activities.

CONCLUSION: These findings indicate that even within a specific cancer the intracellular apoptotic signals engaged by resveratrol are cell type dependent and suggest that such differences may be related to differentiation or lack of differentiation of these cells.

INTRODUCTION

Gastric cancer is a major cause of mortality both in developed and underdeveloped countries because currently available chemotherapeutic regimens are not very effective, resulting in high recurrence rates and poor survival. There is strong evidence that the predominant etiological factors contributing to development of gastric cancer are infections with *H pylori* during early years of life and/or exposure to chemical carcinogens such as those in cigarettes and cured meat^[1]. Identification and eradication of *H pylori* in the world population would be an economically prohibitive undertaking because more than 50% of population over the age of 50 are infected with the bacterium, and eradication would not benefit those with pre-malignant gastric mucosal alterations. However, given the epigenetic origin and prolonged onset of gastric cancer development, the concept of cancer chemoprevention presents an attractive hypothesis to reduce the risk of gastric cancer.

Since apoptosis-inducing compounds control cancer cell proliferation, it is feasible that cancer development may be arrested through molecular intervention with compounds that retard cellular proliferation and induce apoptosis. Trans-resveratrol, a polyphenol found in grapes, wine and peanuts, presents itself as a dietary chemopreventive because numerous studies have demonstrated its ability to suppress proliferation and induce apoptosis in a variety of transformed cells^[2]. We have shown that gastric adenocarcinoma cells respond to resveratrol with inhibition of DNA synthesis, cell cycle arrest, suppressed proliferation, and induction of apoptosis^[3,4], and there is evidence that resveratrol inhibits the growth of transplanted gastric tumor^[5].

There are two major pathways for induction of apoptosis: (1) the extrinsic pathway activated when

extracellular ligands interact with receptors of the TNF family (TNF, FAS, and TRAIL) and (2) the intrinsic pathway, induced by destabilization of mitochondria. Resveratrol up-regulates Fas and Fas-L in gastric adenocarcinoma cells that express p53, whereas only Fas-L becomes up-regulated in cells whose p53 is deleted^[6], suggesting that apoptotic signals engaged by resveratrol within individual gastric carcinoma cell lines may be dependent on p53 status of the cell. Here we explore the action of resveratrol on intracellular apoptotic signals in three different gastric adenocarcinoma cell lines.

MATERIALS AND METHODS

Materials

Resveratrol is a kind gift from Pharmascience, Montreal, Quebec, Canada. The following caspase substrates were purchased from Calbiochem, EMD Biosciences, Inc., San Diego, CA: caspase 1 substrate (Ac-WEHD-pNA), caspase 3 substrate (Ac-DEVD-pNA), caspase 6 substrate (Ac-VEID-pNA), caspase 8 substrate (Ac-IETD-pNA), and caspase 9 substrate (Ac-LEHD-pNA). Cytochrome C Oxidase Assay Kit (CYTOC-OX1) was purchased from Sigma-Aldrich, St. Louis, MO. Antibodies to cytochrome C and lamin B were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA; antibodies to PARP, Bcl-xl, Bax, Bid, and cleaved Lamin A were from Cell Signaling Biotechnology, Beverly, MA; antibody to survivin was from Novus Biologicals, Inc., Littleton, CO; p53 antibody was from PharMingen, Inc./BD Biosciences, San Diego, CA; antibody to β -actin was from Sigma-Aldrich, St. Louis, MO, and antibodies to Bcl-2 and Smac/DIABLO were from Calbiochem/EMD Biosciences, Inc., La Jolla, CA. Chemiluminescence detection system (ECL Western Blotting System) was from Amersham/Pharmacia Biotech, Piscataway, NJ.

Cell culture and cell treatment

Human gastric adenocarcinoma cell lines AGS (ATCC: CLR-1739), SNU-1 (ATCC: CRL-5971) and KATO-III (ATCC: HTB 103) were routinely cultured in RPMI-1640 media supplemented with 100 mL/L fetal bovine serum (FBS), 10 U/mL of streptomycin and 0.25 mg/L of amphotericin B at 37°C in humidified air with 50 mL/L CO₂. Cells were allowed to equilibrate in fresh media for 2-3 h prior to addition of resveratrol which was dissolved in 950 mL/L ethanol. The concentration of ethanol was always maintained at 0.1% in both treated and untreated cells. A stock solution of 100 mmol/L resveratrol was prepared weekly and stored in the dark at -20°C.

Determination of apoptosis

Percent of apoptotic cells was determined using Cell Death Detection ELISA^{PLUS} kit from Roche Applied Science, Indianapolis, IN. Cells (1×10^4 cells/200 μ L) were incubated for designated times with or without 100 μ mol/L resveratrol, and percent of apoptotic cells was calculated based on 100% apoptosis obtained after 48 h of cell exposure to 50 μ mol/L camptothecin.

Caspase activity measurements

Cells were plated in fresh media at 1×10^6 cells/5 mL in 6 well plates, allowed to equilibrate for 3 h, and treated with 100 μ mol/L resveratrol for 24 or 48 h. Vehicle was added to untreated controls that were also cultured for 24 or 48 h. At the end of the specified incubation time, cells were harvested, washed once with 5 mL of PBS and lysed for 30 min at 4°C in lysis buffer A (10 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 0.2 mmol/L sodium vanadate, 2 mmol/L phenylmethyl-sulfonyl fluoride, 0.5% NP-40, and 20 mmol/L NaF). The caspase assay was performed in 96 well plates in a total volume of 100 μ L as follows: to 32 μ L of assay buffer (312.5 mmol/L HEPES, 31.25% glucose, 0.3125% CHAPS) were added 2 μ L of DMSO, 10 μ L of 100 mmol/L DTT, 30 μ g of cell lysate protein, and the volume adjusted with deionized water to 98 μ L. Following addition of 2 μ L of 10 mmol/L fluorogenic peptide substrate, specific for each caspase, the reaction was incubated at 25°C for 4 h after which time absorbance measured at 405 nm on a microplate reader (Molecular Devices Corporation, Sunnyvale, CA). Activity of each caspase activity was calculated from a standard curve of p-nitroaniline (pNA) absorbance at 405 nm and values are expressed as pmoles of pNA generated per mg lysate protein. Protein content of cell lysates was determined by the method of Lowry^[7].

Western blot analysis

Cellular levels of cytochrome C, p53, survivin, Bax, Bcl-2, Bcl-xl, Bid, Smac/DIABLO, cleaved lamin A, lamin B, PARP, and β -actin (used as control for equal protein loading) were determined in untreated and resveratrol treated cell fractions by Western blot detection. To test the action of resveratrol, cells (0.2×10^9 cells/L of media) were treated with 100 μ mol/L resveratrol for 24 or 48 h, after which time they were harvested, washed with PBS, suspended in 0.5 mL of lysis buffer B (10 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 0.2 mmol/L sodium vanadate, 2 mmol/L phenylmethyl-sulfonyl fluoride, 0.5% NP-40, 20 mmol/L NaF, and 2 mg/L leupeptin), and rapidly frozen in liquid nitrogen. Cells were lysed by a freeze-thaw cycle in liquid nitrogen followed by 30 min at 4°C and cell cytosol was prepared from cell lysates by centrifugation at $10\,000 \times g$ for 20 min. Mitochondria were prepared from whole cells by a subcellular fractionation protocol^[8] and protein content of each fraction determined by the Lowry method^[7]. Equal amounts of protein were separated by SDS-PAGE on either 15% resolving Tris-HCl gels for detection of cytochrome C, survivin, Bcl-2, Bcl-xl, Bax, Bid, and Smac/DIABLO or 12% gels for detection of p53, PARP, cleaved lamin A, lamin B, β -actin, followed by transfer to PVDF membranes, and antigen detected by chemiluminescence. Depicted results are representative of at least two individual experiments.

Measurement of cytochrome C oxidase activity

Cytochrome C oxidase activity was measured in cell

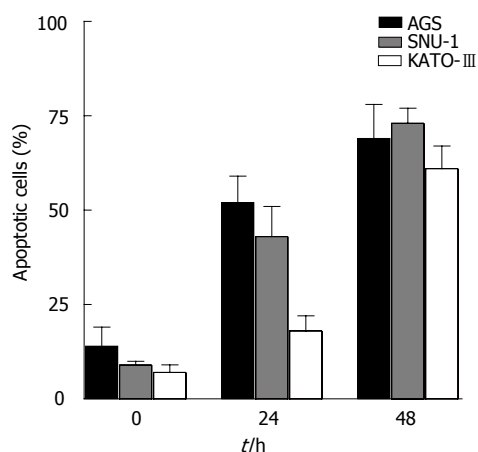


Figure 1 Effect of resveratrol on apoptosis of gastric adenocarcinoma cells.

lysates from untreated and resveratrol treated cells using a commercially available kit from Sigma-Aldrich. Activity of cytochrome C oxidase was determined by measuring the conversion of reduced cytochrome C, which absorbs light at λ_{550} , to its oxidized form that does not absorb light at this wavelength, and values are expressed as ΔA_{550} generated by 35 μ g of lysate protein.

Statistical analysis

Numerical data were analyzed for statistical significance by Student's *t* test, and Results are expressed as the mean \pm SE. Statistical significance is denoted as $P < 0.01$ and $P < 0.05$.

RESULTS

Effect of resveratrol on apoptosis of SNU-1 cells

Gastric adenocarcinoma SNU-1 cells (expressing p53) and Kato-III cells (p53 deleted) respond to resveratrol treatment with decreased proliferation, concentration dependent inhibition of DNA synthesis and cell cycle arrest^[3,4]. To determine whether p53 status of the cell regulates the action of resveratrol on apoptosis, we measured the extent of apoptosis in three gastric adenocarcinoma cells lines: AGS (expressing wild type p53), SNU-1 (expressing p53) and KATO-III. Results show that cell treatment with 100 μ mol/L resveratrol induces a time dependent apoptosis in all three cell lines (Figure 1). Although a small percentage of apoptotic cells was present in the absence of resveratrol, treatment with 100 μ mol/L resveratrol for 24 h resulted in significantly increased accumulation of apoptotic cells, and treatment for 48 h further increasing the percentage of apoptotic cells. After 48 h of treatment with 100 μ mol/L resveratrol nearly 70% of AGS cells, 75% of SNU-1 cells and 62% of Kato-III cells became apoptotic.

Action of resveratrol on nuclear targets of apoptosis

Apoptosis is characterized by loss of nuclear integrity and degradation of DNA and we previously demonstrated that exposure of gastric adenocarcinoma cells to resveratrol results in DNA fragmentation^[3,4]. Here we determined the action of resveratrol on nuclear proteins PARP, lamin

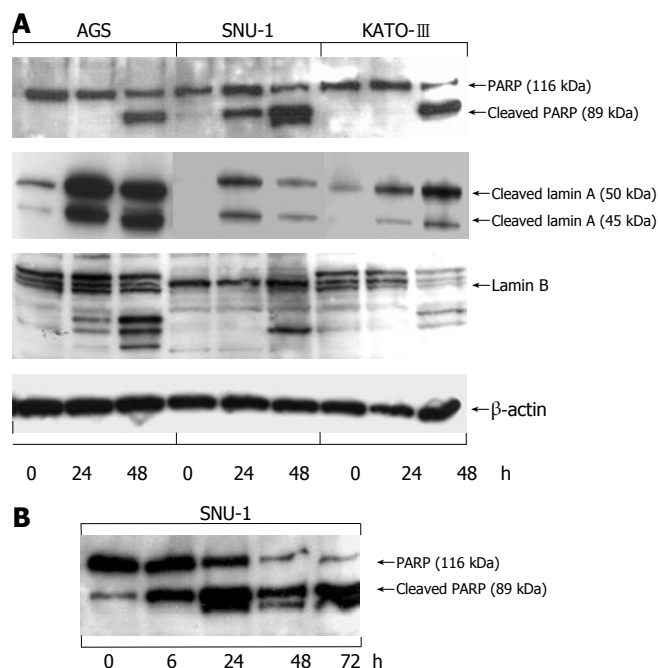


Figure 2 Effect of resveratrol on lamin A, lamin B (A) and PARP (B).

A and lamin B (Figure 2A), and show that resveratrol promotes their cleavage in all three cell lines. Increased levels of lamin A cleavage products were seen in all cells within 24 h of resveratrol treatment, whereas lamin B cleavage products were seen in AGS cells after 24 h, while SNU-1 and Kato-III cells required 48 h of exposure to 100 μ mol/L resveratrol to induce lamin B cleavage. Resveratrol treatment caused cleavage of the 116 kDa PARP to an 89 kDa fragment in all three cell lines although there was a time difference among the individual cell lines. In SNU-1 cells the 89 kDa breakdown product of PARP was detected after 24 h while in AGS and KATO-III cells PARP breakdown product was seen after 48 h. Closer analysis of PARP in SNU-1 cells revealed presence of the 89 kDa fragment within 6 h after cell exposure to resveratrol with further breakdown of the 89 kDa band to a smaller fragment after prolonged treatment and nearly total loss of the 116 kDa PARP protein after 72 h of cell exposure to 100 μ mol/L (Figure 2B).

Action of resveratrol on caspases

Because nuclear proteins become cleaved during resveratrol-induced apoptosis, we investigated whether this action of resveratrol results from activation of caspases. Activities of caspases 1, 3, 6, 8, and 9 were measured in untreated cells and in cells treated for 24 or 48 h with 100 μ mol/L resveratrol. Results show that 100 μ mol/L resveratrol had no effect on caspases 1, 8, and 9, but significantly stimulated caspase 3 activity in AGS and KATO-III cells and caused some activation of caspase 6 in AGS cells after 24 h. Caspase 3 activity in AGS cells was increased after 24 h of treatment and remained elevated after 48 h, whereas in KATO-III cells caspase 3 activity responded to resveratrol only after 48 h. Resveratrol, however, had no effect on caspase 3 activity in SNU-1 cells (Figure 3).

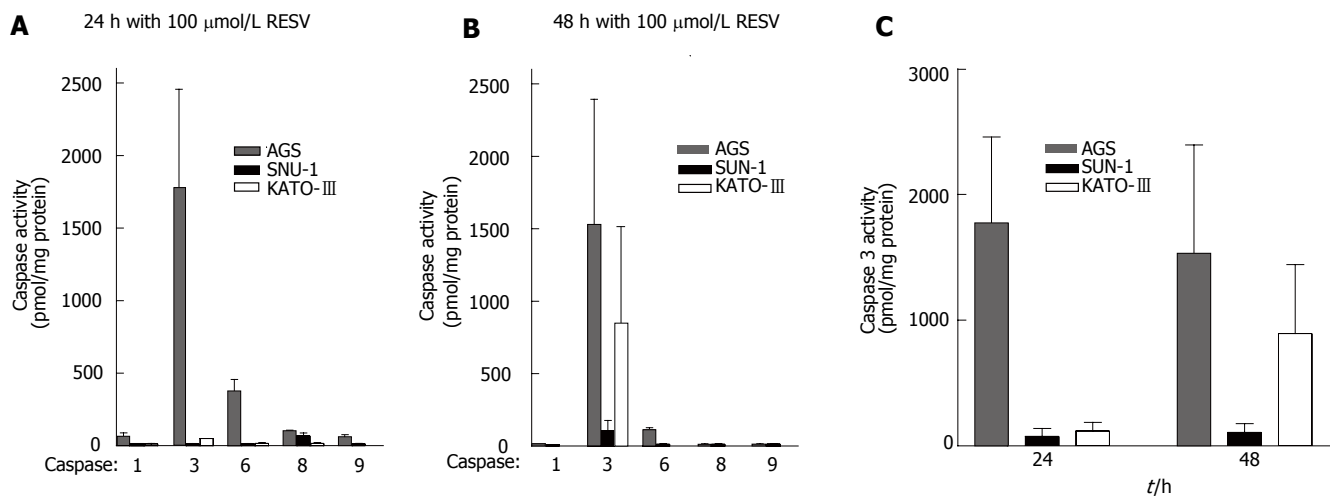


Figure 3 Effect of resveratrol on caspase activity.

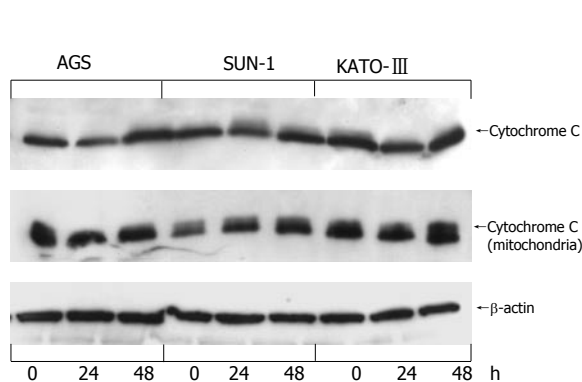


Figure 4 Effect of resveratrol on subcellular distribution of cytochrome C.

Action of resveratrol on subcellular distribution of cytochrome C

Numerous cytotoxic reagents, radiation, and growth factor withdrawal induce apoptosis by promoting release of mitochondrial cytochrome C into cell cytosol. To determine whether resveratrol targets mitochondrial permeability in gastric adenocarcinoma cells we measured distribution of cytochrome C protein between cell cytosol and mitochondria after 24 and 48 h of cell treatment with 100 $\mu\text{mol/L}$ resveratrol. Results indicate that cytochrome C was found to be present in both mitochondria and cytosol of each cell line prior to treatment with resveratrol, and resveratrol treatment had no further effect on redistribution of cytochrome C protein between these two compartments (Figure 4).

Action of resveratrol on cytochrome C oxidase activity

In addition to its role in apoptosis, cytochrome C functions in the respiratory chain as carrier of electrons from flavoproteins to cytochrome oxidase. Since resveratrol is a known antioxidant and inhibits mitochondrial respiratory chain^[9], we inquired whether cytochrome C contributes to the antioxidant potential of resveratrol by measuring cytochrome C oxidase activity in untreated as well as resveratrol treated cells. Results show that resveratrol had a significant stimulatory effect on cytochrome C oxidase activity in KATO-III cells after 24 h of cell treatment, and

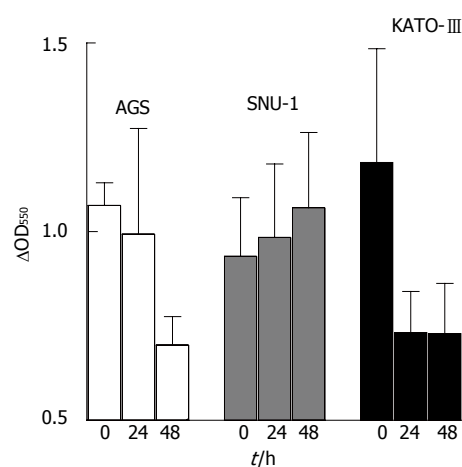


Figure 5 Action of resveratrol on cytochrome C oxidase activity.

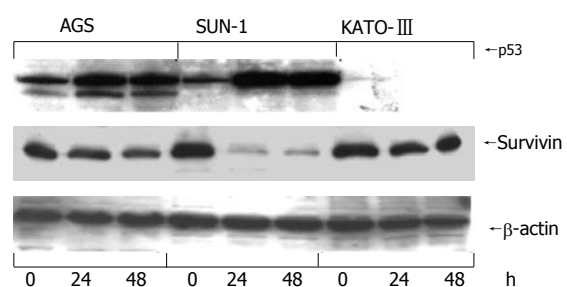


Figure 6 Effect of resveratrol on p53 and survivin.

in AGS cells after 48 h of treatment, but had no effect on cytochrome C oxidase activity in SNU-1 cells (Figure 5).

Action of resveratrol on p53 and survivin levels

Cellular levels of survivin, an inhibitor of apoptosis, have been shown to correlate inversely with expression of the p53 tumor suppressor^[10-12], and we determined the action of resveratrol on survivin and p53 protein levels in AGS, SNU-1 and Kato-III cells. Results presented in Figure 6 indicate that resveratrol up-regulates p53 protein in AGS and SNU-1 cells but has no effect on the p53 status in Kato-III cells. Within 24 h after exposure to 100 $\mu\text{mol/L}$

resveratrol survivin levels in SNU-1 cells became down-regulated. Although AGS cells express p53 and its p53 is up-regulated by resveratrol, levels of survivin in AGS cells were not altered by resveratrol, and resveratrol had no effect on survivin in KATO-III cells.

Action of resveratrol on Bcl-2 proteins

To evaluate the contribution of pro- and anti-apoptotic Bcl-2 proteins in the response of gastric carcinoma cells to resveratrol we measured protein levels of anti-apoptotic Bcl-2 and Bcl-xl and pro-apoptotic Bax, as well as Bid and Smac/DIABLO. Cells were treated with 100 $\mu\text{mol/L}$ resveratrol for 24 or 48 h, sub-cellular fractions prepared as described in Methods, and protein levels determined by Western blotting. Isolated mitochondria were used to determine Bcl-2 and Bcl-xl levels, whereas Bax, Bid and Smac/DIABLO were measured in cell cytosol. Results presented in Figure 7 show that cell treatment with 100 $\mu\text{mol/L}$ resveratrol for up to 48 h had no effect on Bax, Bcl-2, Bcl-xl, and Smac/DIABLO, and did not promote cleavage of cytosolic 22 kDa Bid to the active 15-17 kDa form.

DISCUSSION

A common feature of malignant cells is their ability to proliferate without restraint and, therefore, apoptosis is considered the predominant pathway for elimination of malignant cells with the signals engaged by an apoptotic agent determining its efficacy as a chemopreventive or as an adjuvant to chemotherapy. Previous work from this laboratory has shown that resveratrol-induced engagement of Fas receptor is dependent on the p53 status of the cells. The aim of the present investigation was to determine whether intracellular apoptotic signals engaged by resveratrol are also regulated to some extent by p53. To enhance the signaling response to resveratrol all experiments were performed using 100 $\mu\text{mol/L}$ because this concentration has been repeatedly shown to promote significant apoptotic response in human gastric adenocarcinoma cells^[3-5] and in other malignant cells^[13-15].

One of the differences among the three gastric carcinoma cell lines used in this study is their p53 status: Kato-III cells do not express p53, whereas p53 is present in both AGS and SNU-1 cells. Resveratrol induced a time-dependent apoptotic response in all three cell lines irrespective of their p53 status, and in each cell line resveratrol-induced apoptosis was associated with cleavage of PARP, lamin A and lamin B. Other studies investigating the involvement of p53 in cellular response to resveratrol have also shown that resveratrol induces apoptosis and up-regulates p53 in cells that express this tumor suppressor^[16,17], and that it induces apoptosis in p53 deficient cells^[18,19]. Tumor suppressor p53 exerts control over proliferation and apoptosis by initiating transcriptional activation of specific genes, among them the gene for survivin^[10-12], an inhibitor of apoptosis found to be expressed in all types of malignancies but not in normal, differentiated cells. Patients with gastric cancer express increased abundance of survivin^[20] and survivin expression correlates with p53 accumulation^[18], whereas

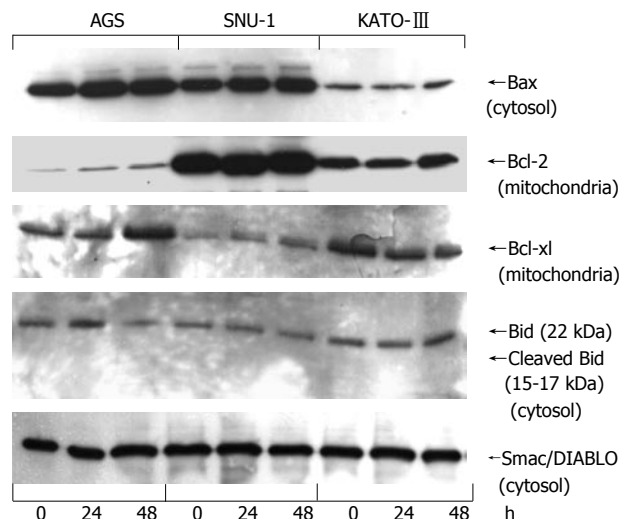


Figure 7 Effect of resveratrol on Bax, Bcl-2, Bcl-xl, Bid and Smac/DIABLO.

suppression of survivin inhibits growth of gastric cancer cells and decreases tumorigenesis^[21]. In our study resveratrol increased p53 levels in both AGS and SNU-1 cells, but only p53-expressing SNU-1 cells responded to resveratrol treatment with loss of survivin suggesting that p53 in AGS cells may have mutations, a common finding in tumor cells.

Since resveratrol is a small, lipophilic molecule it can intercalate within the mitochondrial membrane and directly induce apoptosis through destabilization of mitochondrial membrane. To address this question we determined action of resveratrol action on Bcl-2 family of apoptotic regulators. These proteins control mitochondrial permeability and promote release of mitochondrial cytochrome C. Our findings revealed that resveratrol had no effect on either anti-apoptotic Bax or pro-apoptotic Bcl-2 and Bcl-xl, did not promote cleavage of Bid and had no effect on subcellular redistribution of cytochrome C. On the basis of these results we conclude that resveratrol-induced apoptosis of gastric cancer cells in culture is not an outcome of mitochondrial integrity breakdown. The action of resveratrol on Bcl-2 proteins has been investigated in a number of cells with results supporting a cell type-dependent response. Human leukemia U937 cells readily succumbed to apoptosis after treatment with 100 $\mu\text{mol/L}$ resveratrol, whereas apoptosis was significantly inhibited when these cells were modified to over-express Bcl-2^[22]. In non-Hodgkin's lymphoma and multiple myeloma cell lines Bcl-xl expression was down-regulated by resveratrol^[23], but resveratrol had no effect on Bcl-xl, Bcl-2 nor Bax in other malignant cells^[24], and Bax levels remained unchanged in colorectal carcinoma cells after 24 h of treatment with 40 $\mu\text{mol/L}$ resveratrol^[25]. Since resveratrol caused a decrease in the ratio of Bcl-2/Bax in transplanted gastric tumors and we can only conclude that cells in culture respond differently to resveratrol from primary gastric cancer cells transplanted into nude mice.

Destabilization of mitochondria usually results in release of mitochondrial cytochrome C into cells cytosol, but we found no evidence of involvement of

mitochondria destabilizing Bcl-2 proteins in resveratrol-induced apoptosis of gastric carcinoma cells. However, we did observe presence of cytochrome C in cytosol of untreated as well as resveratrol treated cells. Cytosolic cytochrome C has also been identified within secretory granules of normal rat pancreas and anterior pituitary^[26], and because secretory granules are present in gastric carcinoma cells^[27,28], we addressed the possible role of cytosolic cytochrome C by determining cytochrome C oxidase activity. Our data revealed that resveratrol stimulates cytochrome C oxidase activity in AGS and KATO-III cells, an action that would contribute to the antioxidant potential of these cells. Since transformed cells generate low levels of reactive oxygen that activate gene transcription and stimulate their proliferation^[29,30], antioxidants suppress generation of endogenous reactive oxygen and are considered antiproliferative. We previously demonstrated that resveratrol is antiproliferative and behaves as an antioxidant in gastric adenocarcinoma cells^[3,4]. Our current finding that resveratrol stimulates cytochrome C oxidase is a feasible mechanism for its antioxidant and antiproliferative action toward these. Inhibition of proliferation and induction of apoptosis are known to be closely related events, and when activation of cytochrome C oxidase is coupled with increased caspase 3 activity, as was seen in AGS and KATO-III cells, these events might provide sufficient stimuli to inhibit cellular proliferation and induce apoptosis. Although resveratrol had no effect on either cytochrome C oxidase or caspase 3 in SNU-1 cells, apoptosis in SNU-1 cells probably results from down-regulation of survivin and removal of its protective mechanisms, as was demonstrated for human bladder cells^[31].

In summary, our results reveal that individual gastric carcinoma cell lines respond to resveratrol with engagement of individual apoptotic signals. In p53 expressing SNU-1 cells resveratrol up-regulated p53 and down-regulated survivin, whereas in KATO-III cells and in AGS cells resveratrol stimulated caspase 3 and cytochrome C oxidase activities, enabling suppression of proliferation while stimulating breakdown of nuclear proteins. These findings indicate that even within a specific disease resveratrol can engage alternate apoptotic targets thus providing further evidence that resveratrol can be considered a versatile chemopreventive agent.

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Tyrosine kinase of insulin-like growth factor receptor as target for novel treatment and prevention strategies of colorectal cancer

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Abstract

AIM: To investigate the antineoplastic potency of the novel insulin-like growth factor 1 receptor (IGF-1R) tyrosine kinase inhibitor (TKI) NVP-AEW541 in cell lines and primary cell cultures of human colorectal cancer (CRC).

METHODS: Cells of primary colorectal carcinomas were from 8 patients. Immunostaining and crystal violet staining were used for analysis of growth factor receptor protein expression and detection of cell number changes, respectively. Cytotoxicity was determined by measuring the release of the cytoplasmic enzyme lactate dehydrogenase (LDH). The proportion of apoptotic cells was determined by quantifying the percentage of sub-G1 (hypodiploid) cells. Cell cycle status reflected by the DNA content of the nuclei was detected by flow cytometry.

RESULTS: NVP-AEW541 dose-dependently inhibited the proliferation of colorectal carcinoma cell lines and primary cell cultures by inducing apoptosis and cell cycle arrest. Apoptosis was characterized by caspase-3 activation and nuclear degradation. Cell cycle was arrested at the G1/S checkpoint. The NVP-AEW541-mediated cell cycle-related signaling involved the inactivation of Akt and extracellular signal-regulated kinase (ERK) 1/2, the upregulation of the cyclin-dependent kinase inhibitors p21^{Waf1/Cip1} and p27^{Kip1}, and the downregulation of the cell cycle promoter cyclin D1. Moreover, BAX was upregulated during NVP-AEW541-induced apoptosis, whereas Bcl-2 was downregulated. Measurement of LDH release showed that the antineoplastic effect of NVP-AEW541 was not due to general cytotoxicity of the compound. However, augmented antineoplastic effects were ob-

served in combination treatments of NVP-AEW541 with either 5-FU, or the EGFR-antibody cetuximab, or the HMG-CoA-reductase inhibitor fluvastatin.

CONCLUSION: IGF-1R-TK inhibition is a promising novel approach for either mono- or combination treatment strategies of colorectal carcinoma and even for CRC chemoprevention.

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Key words: Insulin-like growth factor receptor; Tyrosine kinase; Colorectal cancer; Apoptosis; Cell cycle arrest

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the fourth most frequent cause of cancer-related deaths worldwide. Long-term survival of colorectal cancer is related to the stage of disease. Once distal metastases develop the prognosis is poor^[1]. At least 40% of patients with colorectal cancer develop distal metastases and most of them die thereof^[2]. Hence, innovative approaches are urgently needed to improve the treatment of advanced colorectal cancer.

There is evidence that insulin-like growth factor 1 receptor (IGF-1R) may be a promising protein for specific and targeted therapeutic approaches^[3]. Several reports indicate that IGF-1R is overexpressed in the majority (> 90%) of colorectal carcinomas, most likely contributing to the aggressive growth characteristics of these tumors and the poor prognosis^[4,5]. IGF-1R is a tetrameric transmembrane receptor tyrosine kinase (TK) composed of two α and two β subunits. The extracellular α subunit is responsible for ligand binding, whereas the β subunit consists of a transmembrane domain and a cytoplasmic tyrosine kinase domain^[6,7]. The receptor is predominantly activated by IGF-I and II but can also be activated by insulin at a much lower affinity (500-1000 fold less). Ligand binding activates in-

trinsic tyrosine kinase activity, resulting in trans- β subunit autophosphorylation and stimulation of signaling cascades that include IRS-1/PI-3K/PKB/S6K and Grb2/Sos/Ras/MAPK pathways^[8,9].

In general, both IGFs and IGF-1R are involved in the development and progression of several cancers^[8-10]. Activation of IGF-1R by its ligands results in proliferation, survival, transformation, metastasis, and angiogenesis. Hence, abnormal or enhanced expression of IGFs and IGF-1R has been correlated with disease stage, reduced survival, metastasis development and de-differentiation of a broad variety of tumors. Obesity and diabetes are associated with an increased risk of colorectal cancer^[11]. This effect seems to be due to alterations in the metabolism of endogenous hormones, including sex steroids, insulin and also activation of the IGF/IGF-receptor system which further supports the idea of the IGF/IGF-receptor system to be a promising target for colorectal cancer treatment and chemoprevention. Several studies have demonstrated the therapeutic potential of interfering with IGF-1R-mediated signaling in cancer cells *in vitro* and *in vivo*. These approaches include the use of antagonistic IGF-1R antibodies, IGF-1R antisense oligonucleotides, or IGF-1R siRNA^[12-14]. Specific inhibition of IGF-1R TK activity appears to be another promising principle.

Recently, NVP-AEW541, an orally available low-molecular-weight pyrrolo [2, 3-d] pyrimidine derivative, has been introduced as a potent and reversible inhibitor of IGF-1R tyrosine kinase activity^[15]. NVP-AEW541 has been shown to be highly selective for IGF-1R-TK, as compared to both the closely related insulin receptor (InsR) and other tyrosine or serine/threonine kinases. Antitumor activity of NVP-AEW541 has already been demonstrated in fibrosarcomas and breast cancer^[16]. IGFR-TK inhibition has not been evaluated for the treatment of colorectal cancer. Hence, in the present study we examined the antineoplastic potency of NVP-AEW541 both in human colorectal carcinoma cell lines and in primary cell cultures of human CRC, which shows that NVP-AEW541 potently inhibits colorectal cancer growth by inducing apoptosis and cell cycle arrest in human colorectal carcinoma cells.

MATERIALS AND METHODS

Cell lines and drugs

The human colorectal adenocarcinoma cell line HT29 was cultured in RPMI 1640 medium supplemented with 100 g/L fetal bovine serum, penicillin (100 kU/L), and streptomycin (100 mg/L). The human colorectal adenocarcinoma cell line HCT116 was grown in Dulbecco's minimal essential medium containing 100 g/L fetal bovine serum, penicillin (100 kU/L), and streptomycin (100 mg/L). Cells were kept in a humidified atmosphere (50 mL/L CO₂) at 37°C. Cells were incubated with culture medium containing NVP-AEW541 (Novartis, Basel, Switzerland). For combination treatment, cells were incubated simultaneously with NVP-AEW541 and one of the following drugs: 5-fluorouracil (Sigma, Deisenhofen, Germany), SN-38 (Rhone-Poulenc Rorer, Antony, France), cetuximab (Merck KgaA, Darmstadt,

Germany) or fluvastatin (Calbiochem-Novabiochem, Bad Soden, Germany). Stock solutions (in DMSO, stored at -20°C) were diluted to the final concentration in fresh media before each experiment. In all experiments, the final DMSO concentration did not exceed 1 mL/L.

Isolation and establishment of primary cell cultures from human colorectal cancers

Cells of primary colorectal carcinomas from 8 patients (5 males, 3 females, age range 74 \pm 14 years) were isolated from endoscopically taken biopsies as previously described^[17]. The human tumor material was used according to the standards set by the Ethical Committee of the Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany. Cell preparation was performed by incubation (30 min/RT) with a solution containing 0.5 g/L trypsin, 0.2 g/L EDTA and 1 g/L collagenase. The isolated human colorectal carcinoma cells were maintained in Earle's 199 medium supplemented with 200 g/L FBS, 2 mmol/L L-glutamine, 20 g/L Biotect protective medium, penicillin (100 kU/L), streptomycin (100 mg/L), 10 g/L amphotericin B, and incubated at 37°C in a humidified atmosphere (50 mL/L CO₂).

Analysis of growth factor receptor expression

For analysis of growth factor receptor protein expression, cells were immunostained as previously described^[18,19]. In brief, samples were fixed, permeabilized, and subsequently incubated with a polyclonal anti-EGFR or IGF-1R antibody (5 mg/L, Santa Cruz Biotechnology, Palo Alto, CA), or isotypic control rabbit IgG1 (DAKO, Hamburg, Germany). Cells were then incubated with a secondary FITC-labeled goat-anti rabbit IgG antibody (5 mg/L, BD Pharmingen, Heidelberg, Germany). Fluorescence was detected by flow cytometry on a FACSCalibur (Becton Dickinson, Heidelberg, Germany) and analyzed using CellQuest software.

Determination of cell number

Cell number was evaluated by crystal violet staining as previously described^[20]. In brief, cells in 96-well plates were fixed with 10 mL/L glutaraldehyde and then stained with 1 g/L crystal violet. The unbound dye was removed by washing with water. Bound crystal violet was solubilized with 2 g/L Triton X-100. Light extinction increasing linearly with the cell number was analyzed at 570 nm using an ELISA reader.

Determination of cytotoxicity

Cells were incubated with 0-10 μ mol/L NVP-AEW541 for 1, 6, 12 and 24 h. Release of the cytoplasmic enzyme lactate dehydrogenase (LDH), indicating cytotoxicity, was measured using a colorimetric kit from Roche (Roche Diagnostics, Mannheim, Germany) as described previously^[21,22]. Background release from untreated cells was subtracted. Maximum release was measured after adding 2 g/L Triton X-100 to untreated cells. For determinations, LDH assay reagent was added to sample supernatants and incubated for 30 min at room temperature in dark. Absorbance was measured at 490 nm (reference wavelength 690 nm).

Detection of apoptosis

Preparation of cell lysates and determination of caspase-3 activity were performed as previously described^[23]. The activity of caspase-3 was calculated from the cleavage of the fluorogenic substrate DEVD-AMC (Calbiochem-Novabiochem, Bad Soden, Germany). The proportion of apoptotic cells was determined by quantifying the percentage of sub-G1 (hypodiploid) cells after flow cytometric analysis of isolated propidium iodide-stained nuclei^[24].

Cell cycle analysis

Cell cycle analysis was performed by the method of Vindelov and Christensen^[25]. Cells were trypsinized, washed, and the nuclei were isolated using CycleTest PLUS DNA Reagent Kit (Becton Dickinson). DNA was stained with propidium iodide according to the manufacturer's instructions. DNA content of the nuclei was detected by flow cytometry and analyzed using CellFit software (Becton Dickinson).

Western blotting

Western blotting was performed as previously described^[23]. Blots were blocked in 50 g/L non-fat dry milk for 1 h, and then incubated at 4°C overnight with anti-phospho-Akt, Akt (both 1:1000, Cell Signaling, Beverly, MA), Bax (1:1000, Santa Cruz Biotechnology, CA), Bcl-2 (1:200, Novo Castra Laboratories, Newcastle upon Tyne, UK), COX-2 (1:200), cyclin D1 (1:100), p21^{Waf1/Cip1} (1:200), IGF-1R (1:200), phospho-ERK1/2, ERK1/2 (1:1000, Santa Cruz Biotechnology, CA), or p27^{Kip1} (1:2500, Becton Dickinson). β -actin (1:5000, Sigma) served as a loading control.

Statistical analysis

If not stated otherwise, means of four independent experiments \pm SE were shown. Individual drug treatment was compared by the unpaired, two-tailed Mann-Whitney *U*-test. *P* < 0.05 was considered statistically significant.

RESULTS

Expression of IGF-1R in colorectal carcinoma cells

Expression of IGF-1R and epidermal growth factor receptor (EGFR) was investigated in human colorectal carcinoma cells. Protein expression of IGF-1R was detected in both cell lines. In addition, expression of EGFR protein was detected in both cell lines (Figure 1A). IGF-1R protein expression of NVP-AEW541-treated colorectal cancer cells was determined by Western blotting. HT-29 cells incubated with NVP-AEW541 (0–10 μ mol/L) for 48 h did not abolish the expression of IGF-1R. By contrast even after treatment with 10 μ mol/L of NVP-AEW541, a robust expression of IGF-1R protein could still be observed (Figure 1B). IGF-1R and EGFR expression was confirmed in the investigated 8 primary colorectal cancer cultures by RT-PCR using established primers (not shown)^[18,22].

NVP-AEW541-induced growth inhibition of colorectal carcinoma cells

Cell number changes caused by IGF-1R-TK inhibition

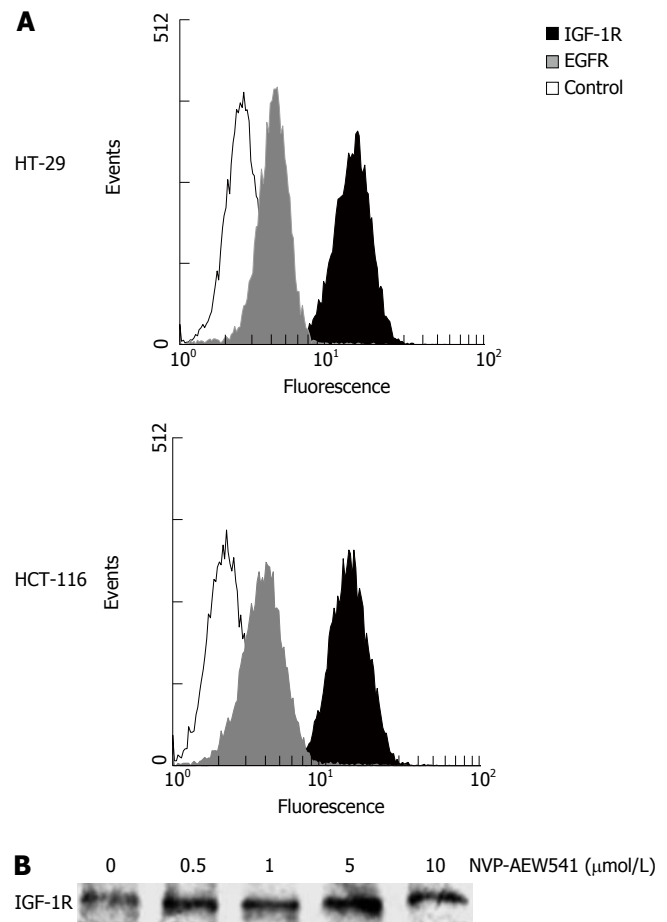


Figure 1 Flow cytometric analysis of IGF-1R and EGFR protein expression in HT-29 (A) and HCT-116 (B) cells. Cells were stained with antibodies against either IGF-1R (black areas) or EGFR (grey areas). Black lines: isotypic controls.

with NVP-AEW541 were studied by crystal violet assays. NVP-AEW541 time- and dose-dependently inhibited the growth of HT-29 and HCT-116 cells (Figure 2A and B). The IC₅₀ values of NVP-AEW541 were 1.7 ± 0.4 μ mol/L (HT-29) and 2.5 ± 0.4 μ mol/L (HCT-116), as determined after 4 d of incubation.

In line with our findings in permanent cell lines, NVP-AEW541 treatment (0–5 μ mol/L) reduced the cell number of primary cultures of human colorectal carcinomas in a dose-dependent manner. After 3 d of incubation a cell number reduction of $47.3\% \pm 2.4\%$ was detected by direct cell counting in six NVP-AEW541-sensitive primary culture preparations. Two out of the investigated 8 primary cultures displayed only a weak growth inhibition of $12\% \pm 4\%$. In treatment-sensitive primary cultures NVP-AEW541 also altered the morphology of the remaining cells, which appeared shrunken and flat. Propidium iodide-positive staining of primary culture cells revealed that NVP-AEW541 treatment led to a loss of cell membrane integrity indicating cell death or that these cells were in the process of dying, respectively (Figure 2C).

Antineoplastic potency of NVP-AEW541 in combination with cytostatics, cetuximab or the HMG-CoA reductase inhibitor fluvastatin

To test whether combination treatment of NVP-AEW541

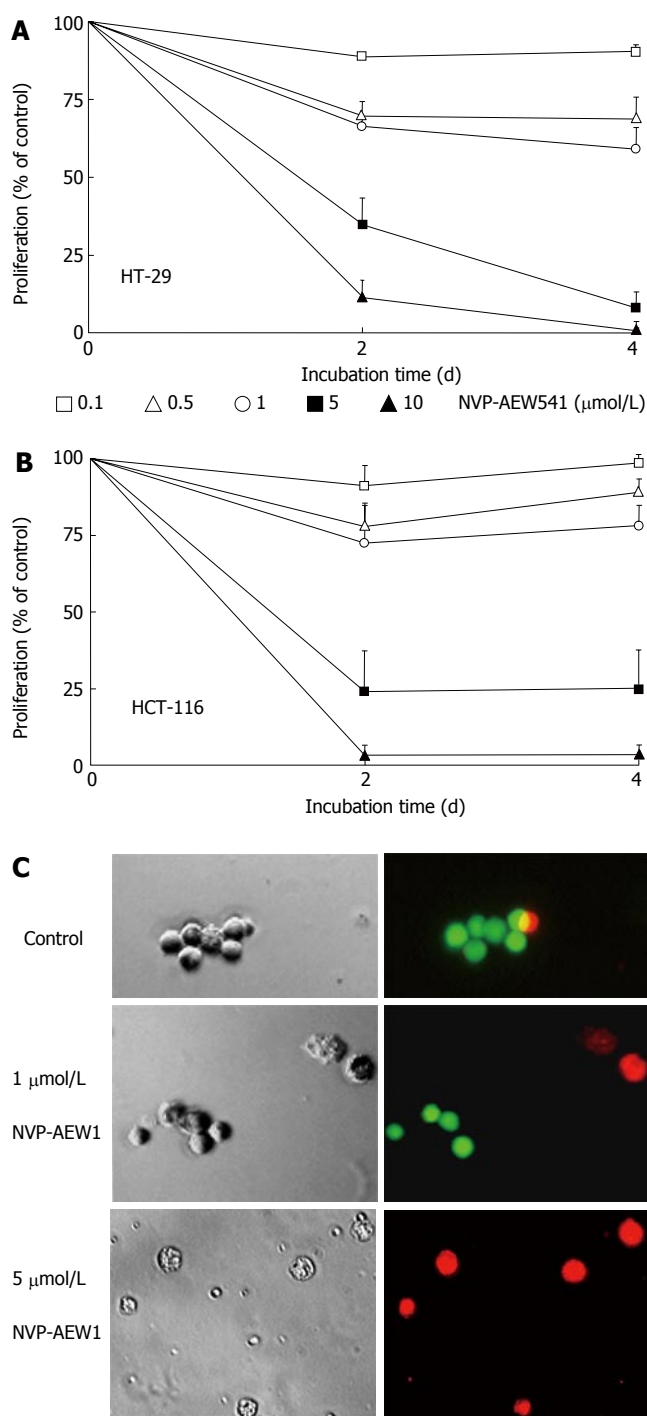


Figure 2 Effects of NVP-AEW541 on HT-29 (A) and HCT-116 (B) cell growth as well as induction of cell death and morphological changes of isolated primary colorectal cancer cells (C). After 4 d of incubation with rising concentrations of NVP-AEW541, the number of HT-29 (A) and HCT-116 (B) cells decreased by > 95%, as determined by crystal violet staining (mean \pm SE, $n = 4$). In both cell lines statistical significance ($P < 0.05$) of growth inhibition by NVP-AEW541 was shown for concentrations of 0.5-10 $\mu\text{mol/L}$. After 3 d of incubation with 0-5 $\mu\text{mol/L}$ NVP-AEW541, the induction of cell death and morphological changes of isolated primary colorectal cancer cells was determined by Live/Dead-fluorescence microscopy (C). Viable cells are stained green, while cells with impaired cell membrane appear red. Phase-contrast images and corresponding fluorescence micrographs of a representative preparation (out of 6 NVP-AEW541-sensitive primary cell cultures) are depicted.

with either 5-fluorouracil (5-FU), SN-38, or whether the humanized monoclonal anti-EGFR antibody cetuximab may lead to additive antiproliferative effects, HT29 and

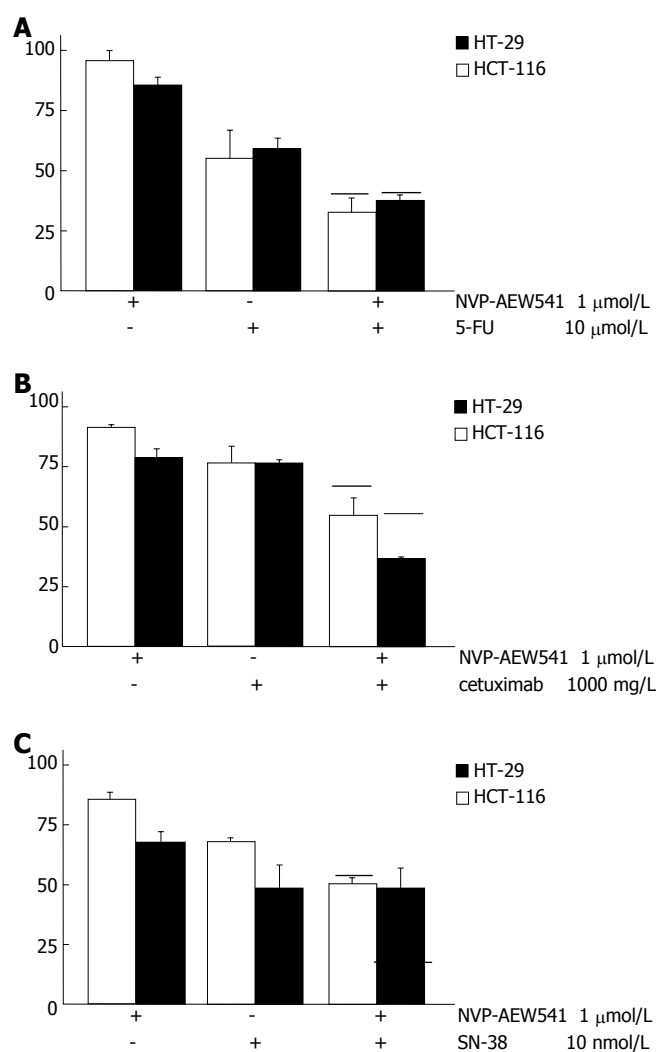


Figure 3 Augmented growth inhibition of combination treatment with NVP-AEW541 plus either 5-FU (A), or SN-38 (B) or cetuximab (C) (mean \pm SE, $n = 4-6$). A: Combination treatment with sub-IC₅₀ concentrations of NVP-AEW541 plus 5-FU led to synergistic growth inhibition of colorectal cancer cells; B: Combination treatment with sub-IC₅₀ concentrations of NVP-AEW541 plus the humanized EGFR-antibody cetuximab resulted in slightly over-additive antiproliferative effects; C: Co-treatment with sub-IC₅₀ concentrations of NVP-AEW541 and SN-38 resulted in additive growth inhibition of HCT-116 cells, while no additive growth inhibition was detected in HT-29 cells. Black bars indicate the values of the calculated additive growth inhibition. Data are given as percentage of untreated controls.

HCT-116 cells were co-treated for 96 h with sub-IC₅₀ concentrations of NVP-AEW541 (1 $\mu\text{mol/L}$) plus either 5-FU (10 $\mu\text{mol/L}$), or cetuximab (1000 mg/L), or SN-38 (10 nmol/L). In both colorectal carcinoma cell models combination treatment with NVP-AEW541 plus either 5-FU (Figure 3A) or cetuximab (Figure 3B) resulted in additive or even over-additive growth inhibitory effects. When NVP-AEW541 was combined with SN-38, additive antiproliferative effects were only observed in HCT-116 cells (Figure 3C). We additionally investigated the anti-proliferative potency of the HMG-CoAR inhibitor fluvastatin, either alone or in combination with NVP-AEW541 in colorectal carcinoma cells (Figure 4). Fluvastatin (0-50 $\mu\text{mol/L}$) caused a dose-dependent growth inhibition of more than 80% both in HCT-116 cells and in HT-29 cells. Combinations of sub-IC₅₀ concentrations of fluvastatin and NVP-AEW541 resulted in an additive growth inhibi-

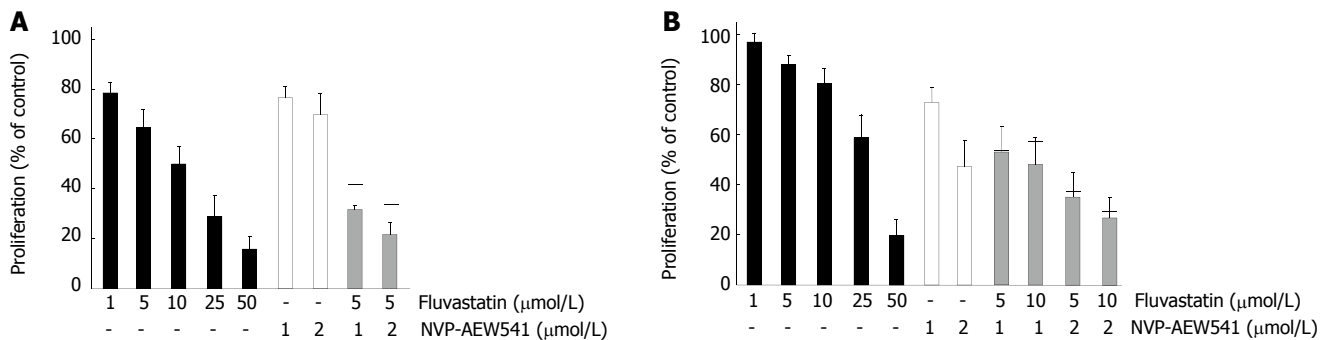


Figure 4 Additive growth inhibition by NVP-AEW541 plus fluvastatin (mean \pm SE, $n = 3-5$). Fluvastatin (1-50 $\mu\text{mol/L}$) induced a dose-dependent growth inhibition of HCT-116 (A) and HT-29 (B) cells by $> 80\%$ when applied for 3 d. Moreover, combination treatment with sub-IC₅₀ concentrations of fluvastatin and NVP-AEW541 led to (over-)additive growth inhibition after 3 d of treatment. Black bars indicate the values of the calculated additive growth inhibition.

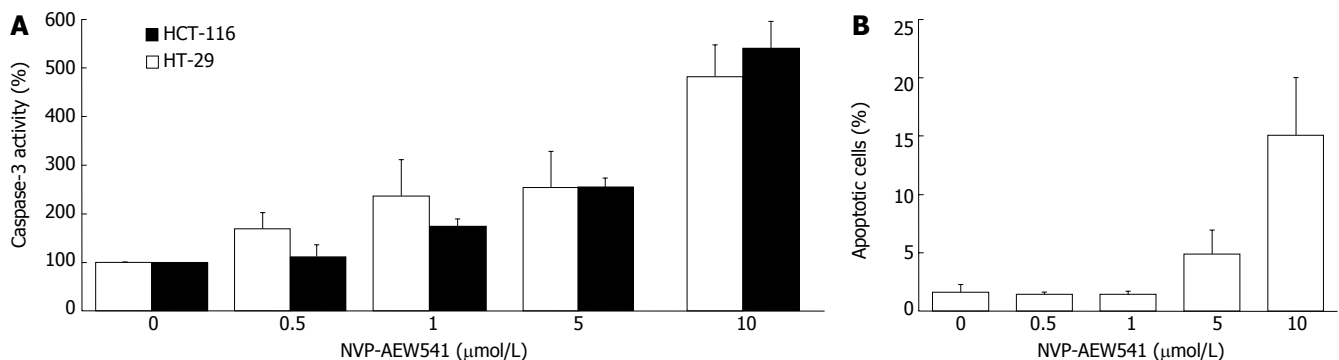


Figure 5 Apoptosis induction by NVP-AEW541 (mean \pm SE, $n = 4$). **A:** After HT-29 and HCT-116 cells were incubated with 0.5-10 $\mu\text{mol/L}$ NVP-AEW541 for 24 h, a dose-dependent induction in caspase-3 activation was observed; **B:** After 72 h of NVP-AEW541 treatment an increased proportion of apoptotic cells measured as subdiploidy was observed in HT-29 cells. $^aP < 0.05$ vs untreated controls.

tion of either colorectal cancer model.

NVP-AEW541-induced apoptosis in colorectal cancer cells

NVP-AEW541 dose-dependently induced a significant increase of caspase-3 activity after 24 h of incubation (Figure 5A). Compared to control cells, an increase of up to 500% was observed. The dose-dependent induction of apoptosis by NVP-AEW541 became also apparent by flow cytometrically monitoring nuclear degradation after 72 h of treatment (Figure 5B).

NVP-AEW541-induced cell cycle arrest in colorectal carcinoma cells.

To test whether an induction of cell cycle arrest contributed to the antiproliferative potency of NVP-AEW541 in colorectal carcinoma cells, we performed cell cycle analyses. NVP-AEW541 dose-dependently arrested HT-29 and HCT-116 cells in the G₁/G₀ phase of the cell cycle after 24 h of treatment, thereby decreasing the proportion of cells in the S and G₂/M phases (Figure 6).

Cytotoxicity of NVP-AEW541

Cytotoxicity of NVP-AEW541 was determined by measuring LDH release. HT29 and HCT-116 cells incubated with 1-10 $\mu\text{mol/L}$ NVP-AEW541 for 1, 6 or 12 h did not result in a measurable increase in LDH release, indicating that NVP-AEW541 did not directly affect cell membrane

integrity. After 24 h of incubation a slight but not significant increase in LDH-release of about 3% was observed at 10 $\mu\text{mol/L}$ NVP-AEW541, indicating that even at high concentrations NVP-AEW541 did not cause immediate necrotic/cytotoxic effects in colorectal cancer cells (data not shown).

NVP-AEW541-induced modulation of cell cycle and apoptosis-related signaling molecules

The effects of NVP-AEW541 on the phosphorylation of both ERK1/2 and its upstream regulator Akt/PKB were investigated to elucidate the signaling pathways modulated by IGF-1R-TK inhibition. NVP-AEW541 treatment dose-dependently decreased the phosphorylation of both mitogenic and antiapoptotic ERK1/2 MAPK as well as Akt/PKB (Figure 7A). Next, the expression of cell cycle-related proteins was investigated to explore the pathway downstream of NVP-AEW541-induced dephosphorylation of Akt/PKB and ERK1/2, known to be causative for cell cycle arrest. NVP-AEW541 decreased the expression of cyclin D1 but increased the expression of the CDK inhibitors p21^{Waf1/CIP1} and p27^{Kip1}. These data suggest that NVP-AEW541-induced cell cycle arrest was mediated by p21^{Waf1/CIP1} and p27^{Kip1} induction, resulting in a decrease of cyclin D1.

To survey the proapoptotic signaling pathways modulated by IGF-1R-TK inhibition with NVP-AEW541 in CRC

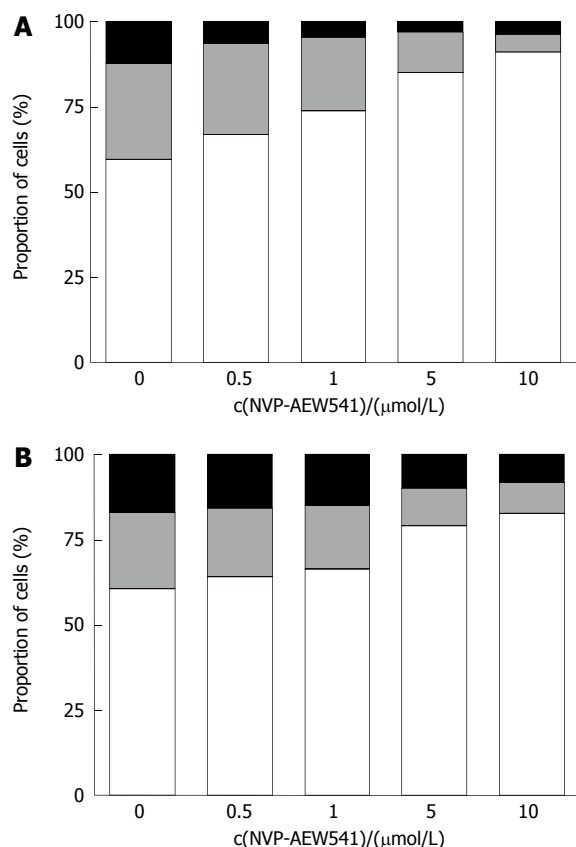


Figure 6 Effects of NVP-AEW541 on the cell cycle of colorectal carcinoma cells (means \pm SE, $n = 4$). After 24 h incubation with NVP-AEW541, a dose-dependent accumulation of HT-29 (A) and HCT-116 (B) cells was observed in the G0/G1 phase of the cell cycle (white bars). Proportion of cells in the S and G2/M phase (grey and black bars) decreased. $^aP < 0.05$ vs untreated controls.

cells, we also investigated the effects of NVP-AEW541 on the expression pattern of Bcl-2 and Bax. Treatment with NVP-AEW541 dose-dependently increased the expression of the proapoptotic Bax protein, while the expression of the antiapoptotic protein Bcl-2 slightly decreased. Finally, it was proved that NVP-AEW541 could downregulate the expression of cyclooxygenase 2 (COX-2) in colorectal cancer cells (Figure 7B).

DISCUSSION

Treatment options for advanced colorectal cancer (CRC) are unsatisfactory. Thus, there is a strong need for effective novel treatment strategies of CRC. A novel approach for CRC treatment may be the interruption of IGF/IGF-receptor signaling system, which is known to have strong stimulatory effects on cancer cell growth. The protective and mitogenic effects of the IGF/IGF-receptor system involve the constitutive activation of antiapoptotic proteins as well as cell cycle promoting signaling. A tight association between IGF-receptor signaling and regulation of cell growth and apoptosis in CRCs which commonly overexpress IGF-receptors has been described^[4,5,26].

Most fast growing cancers produce and release growth factors and thereby auto-stimulate their growth. This also holds true for CRC in which epidermal growth factor (EGF), transforming growth factor α (TGF- α), insulin-like growth factor (IGF) and vascular endothelial growth

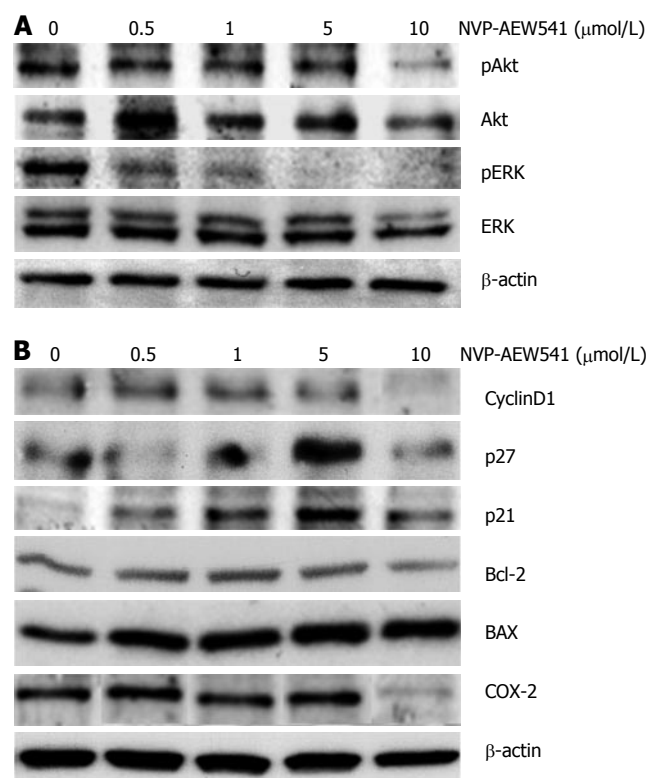


Figure 7 Effects of NVP-AEW541 on the expression and phosphorylation of apoptosis- and cell cycle- related proteins. Modulation of protein phosphorylation or protein expression by NVP-AEW541 treatment was analyzed by Western blotting. **A:** NVP-AEW541 treatment (24 h) induced a dose-dependent dephosphorylation of mitogenic ERK1/2; **B:** NVP-AEW541 dose-dependently increased the expression of the proapoptotic Bax protein, while the expression of the antiapoptotic protein Bcl-2 slightly decreased. The cell cycle promoter cyclin D1 was down-regulated by NVP-AEW541 treatment, while the cell cycle inhibitors p21^{Waf1/Cip1} and p27^{Kip1} were up-regulated. Moreover, COX-2 expression was down-regulated by NVP-AEW541. Expression of β -actin was used as a loading control.

factor (VEGF) are produced and secreted to promote CRC growth^[27]. Therefore, the antiproliferative effects of the novel IGF-1R tyrosine kinase inhibitor NVP-AEW541 were investigated under serum-containing conditions (e.g. in the presence of growth factors like EGF, IGF and TGF- α). Our study demonstrated that inhibition of IGF-1R tyrosine kinase activity by NVP-AEW541 might be suitable for novel targeted therapy of CRC. By abrogating the protective and mitogenic effects of IGF-R signaling, CRC cell growth was potently inhibited by NVP-AEW541. The antineoplastic effects of NVP-AEW541 were based on a pronounced induction of cell cycle arrest and apoptosis. Accompanying or dose-limiting cytotoxicity was not observed, underlining the specific mode of action of the drug. Our findings are in line with recent observations on IGF-R inhibition in other cancer models. Although applying other approaches for IGF-R inhibition, these studies have also shown the strong antineoplastic potency of the interruption of IGF-R signaling without dose-limiting toxicity in several other cancer models^[16,28,29].

Re-initialisation of apoptosis and induction of cell cycle arrest are valuable features for a successful anti-cancer agent. On the one hand, apoptotic cell death is not accompanied with undesired immunological reactions, which occur upon treatment with unspecific and cytotoxic

agents. On the other hand, apoptosis-sensitization and cell cycle arresting effects offer possibilities for powerful combination treatments. In this respect, we investigated combination treatments of NVP-AEW541 and clinically relevant cytostatics with different modes of action. The tested combinations of DNA strand-breaking pyrimidine analog 5-fluorouracil (5-FU) and topoisomerase-I inhibitor SN-38, except for the combination of NVP-AEW541 and SN-38 in HT29 cells, resulted in additive antiproliferative effects. The additive antineoplastic potency appeared to be independent of the mode of action of the respective cytostatic drug. Our data support the notion that NVP-AEW541 is a promising new agent for potentiation of antitumor efficacy of the established cytostatic CRC treatment.

It has been pointed out that the antiapoptotic potency of IGF/IGFR-signaling might interfere with strategies that target other tyrosine kinases such as EGFR-TK. Hence, the antineoplastic potency of EGFR blockade may well be underestimated when examined under conditions where IGF-1R is fully functional. IGF-1R is capable of transactivating EGFR-TK and abrogating the antiproliferative effects of EGFR-antibody treatment^[30-32]. As the CRC cell models used in this study were shown to express both IGF-1R and EGFR, we studied the effects of targeting both growth factor receptors by combination treatment. Treating CRC cells with sub-IC₅₀ concentrations of both NVP-AEW541 and humanized anti-EGFR-antibody cetuximab enhanced the antiproliferative effect as compared to the effect of either agent alone. Thus, NVP-AEW541 qualifies as a promising substance for combination treatment strategies to overcome the compensatory effects of mitogenic crosstalks between IGF-1R and EGFR in CRC.

Drug resistance is one of the major problems of chemotherapy. Potential mechanisms of drug resistance include the activation of Ras/Raf/Mek/ERK signal transduction cascade and the increase of cholesterol levels in cancer cells, both being controlled by isoprenoids^[33]. The production of isoprenoids is catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR) which may therefore be a rational molecular target for innovative antineoplastic treatment of colorectal cancer. Fluvastatin is an effective inhibitor of HMG-CoAR and has already been shown to inhibit tumor cell growth^[34]. In the present study, we have demonstrated the antineoplastic effect of fluvastatin alone and in combination with NVP-AEW541 in colorectal cancer cells and the antiproliferative effect of NVP-AEW541 augmented by fluvastatin, suggesting that combining NVP-AEW541 and fluvastatin may be a promising approach for dual targeting treatment strategies in colorectal cancer disease.

The mechanisms underlying the antiproliferative action of NVP-AEW541 in colorectal cancer cells were further characterized. NVP-AEW541 induced cell cycle arrest in the G1/G0-phase in both CRC cell lines, suggesting that the drug acts at the G1/S checkpoint. A G1/S cell cycle arrest induced by inhibition of IGFR signaling has been described in other fast growing cancers^[35,36]. Moreover, we observed a definite rise in apoptotic cells after treatment with NVP-AEW541. While the induction of apoptosis

is a well-known effect occurring upon inhibition of IGFR-signaling, the underlying mechanisms have been poorly characterized^[37-39]. Our present results suggest that activation of caspase-3 is involved in NVP-AEW541-induced apoptosis of CRC cells. The proapoptotic protein BAX was upregulated during NVP-AEW541-induced apoptosis, while Bcl-2, the protective and antiapoptotic counterpart of Bax, was downregulated. Our findings suggest that involvement of mitochondrial pathways leads to NVP-AEW541-mediated apoptosis.

As activated IGF-1R induces the Ras-Raf-MEK-ERK signaling pathway with subsequent induction of cyclin D1 expression, we analyzed NVP-AEW541-induced changes of Akt/PKB, ERK1/2 activity and p21^{Waf1/CIP1}, p27^{Kip1} as well as cyclin D1 expression as previously described^[40]. In agreement with previous observations in non-colorectal tumor models^[16], we found that NVP-AEW541 could dephosphorylate IGF-1R as well as ERK1/2 MAPK and AKT/PKB of colorectal cancer cells.

IGF-IR has at least three survival signals that are able to protect cancer cells from apoptosis, namely PI-3K/AKT and MAPK/ERK signaling pathways, and a third one that results in the mitochondrial translocation of Raf 1, the so called 14-3-3 pathway^[41]. Simultaneous inactivation of two of these pathways is required to inhibit IGF-1R capacity of protecting cells from apoptotic injuries^[42]. By showing the simultaneous inactivation of AKT/PKB and ERK1/2 MAPK by NVP-AEW541, we hypothesize that blockade of these two survival pathways is directly involved in the successful inhibition of colorectal cancer cell growth by NVP-AEW541.

Defective function of cell cycle regulators is a main cause for tumor development and progression. For example, the cell cycle promoter cyclin D1 is frequently overexpressed in CRC. Successful therapeutic strategies have to balance or bypass the impaired signaling. In the present study, NVP-AEW541 treatment raised the expression of the cell cycle-inhibiting molecules p21^{Waf1/CIP1} and p27^{Kip1}, while it decreased the expression of cyclin D1. Finally, the expression of COX-2 which is known to be upregulated during colorectal carcinogenesis and plays an important role in colorectal cancer growth, was suppressed by IGFR-TK inhibition as previously described^[43]. Thus, NVP-AEW541-induced COX-2 suppression may well contribute to its antineoplastic potency in colorectal cancer. Since both COX-2 and IGFR are up-regulated during colorectal carcinogenesis, NVP-AEW541 appears to be a promising chemopreventive agent in patients at risk for CRC.

Primary cell cultures of human colorectal cancers were established as a tool to design a rational individual medical treatment of an individual patient. The primary goal was to study NVP-AEW541's antineoplastic potency in a bench to bedside approach, as permanent cell lines may represent well-suited but nevertheless non-representative models of colorectal cancers. Moreover, chemosensitivity testing of primary cultures was performed to establish a new method for predicting the response of an individual patient to a certain drug. Attempts to predict individual responses have already been undertaken for breast cancer and colorectal cancer, respectively^[44,45]. Such an approach

may pave the way to an individualized medical treatment of cancer patients. The importance of predictive testing is further supported by our finding that two out of the tested eight primary cell cultures showed only a weak response to NVP-AEW541 treatment. Nevertheless, NVP-AEW541 is a promising compound for future colorectal cancer treatment, as 75% of the investigated primary colorectal cancers could be effectively treated with the drug.

In conclusion, the IGFR-TK inhibitor NVP-AEW541 potently inhibits the growth of human colorectal cancer cells by inducing both cell cycle arrest and apoptosis without eliciting unspecific cytotoxicity. Furthermore, the compound is well-suited for combination treatment approaches. Thus, inhibition of IGFR-signaling by NVP-AEW541 is a promising targeted anticancer strategy for colorectal carcinoma and should be tested in future clinical trials. Moreover, investigations should be pursued to modulate the IGF/IGFR system as a possible means of chemoprevention of colorectal cancer in patients at risk.

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COLORECTAL CANCER

Hemoglobin induces colon cancer cell proliferation by release of reactive oxygen species

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Abstract

AIM: To study whether hemoglobin could amplify colon cancer cell proliferation *via* reactive oxygen species (ROS) production.

METHODS: Colon cancer cell line HT-29 was grown in the conventional method using RPMI1640 media. The viability of the cells was measured using the colorimetric MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay after adding hemoglobin. We determined reactive oxygen species levels to be indicators of oxidative stress in HT 29 cell lines with and without hemoglobin and/or 5-fluorouracil (5-FU), 5'-deoxy-5-fluorouridine (5-DFUR) using fluorometric dichlorofluorescein diacetate (DCFH-DA) assay.

RESULTS: Cellular proliferation was increased with hemoglobin in a concentration-dependent manner. A significant increment on ROS levels was found in HT 29 cells following hemoglobin incubation. The cytotoxic effects of 5-FU and 5-DFUR were significantly blunted by administration of hemoglobin. There was a slight increase of peroxiredoxin 1, superoxide dismutase 1 concentration according to different hemoglobin concentrations.

CONCLUSION: Hemoglobin has a cellular proliferative effect on HT-29 colon cancer cell line by production of ROS. Also, hemoglobin abates cytotoxic effects of chemotherapeutic agents such as 5-FU and 5-DFUR.

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Key words: Colon cancer; Hemoglobin; Reactive oxygen species

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INTRODUCTION

Colon cancer is an important public health issue^[1]. There are nearly one million cases of colon cancer diagnosed worldwide each year. The increasing trend of this cancer is prominent in Asian countries, including Korea^[2,3]. Until the present day, scientists have made an intensive effort to find a provocative factor of this major cancer. Many epidemiologic studies indicate that a western style diet is associated with a high incidence of colon cancer^[4-6]. An especially high protein consumption as in the western-style diet is regarded as a major factor in inducing colon cancer. There is consistent evidence that high meat consumption, in particular red meat, confers an increased risk of this cancer. However, recent large prospective epidemiologic studies that hypothesized a strong relationship between red meat consumption and colorectal cancer development have revealed inconsistent results.

Hemoglobin is a complex of heme and globin, which contribute an important role of oxygen delivery processes to individual tissues^[7]. Intake of these particular molecules contributes nutritional buildup as an iron and protein supplementation. Hemoglobin inside food is already in an oxidized form, and so cannot be used as an oxygen delivery porter. Recently, interesting results about the carcinogenic effects of dietary haemin were reported, documenting that dietary haemin increases the number of aberrant crypt foci in rat colon mucosa^[8-10]. It is important to understand how reactive oxygen species (ROS) are formed in the gut lumen and which biological potency they may have, since intracellular reactions with active oxygen can result in the initiation and progression of carcinogenesis by induction of gene mutations, chromosomal damage and cytotoxic effects^[11-13]. Furthermore, active oxygen regulates expression of genes active during cell differentiation and growth and therefore, probably plays an important role in the promotion phase of tumor generation. In the colon, iron is expected to increase the production of ROS from peroxides via the Fenton reaction, which may be the cause of cellular toxicity and even pro-mutagenic lesions^[14].

The aim of this study was to investigate whether hemoglobin could be classified as a proliferative agent for colon cancer cells by causing reactive oxygen species

release. For this purpose, we have studied the cellular viability of differentiated colon cell line HT29 after administration of hemoglobin at different concentrations. ROS production was investigated in each step. Additionally, we examined the protective effect of hemoglobin on the cytotoxicity of chemotherapeutic agents.

MATERIALS AND METHODS

Reagents and antibodies

Human hemoglobin, 5-fluorouracil (5-FU), 5'-deoxy-5-fluorouridine (5-DFUR) were obtained from Sigma (St. Louis, MO, USA). 2', 7'-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Calbiochem (Meudon, France). Antibodies to human peroxiredoxin 1 and superoxide dismutase 1 were purchased from Labfrontier (Seoul, Korea).

Cell culture

The human colon cancer cell line HT-29 and Lovo was established from Korean Cell Line Bank. To compare the effect on normal fibroblast, we used CCD-33Co normal colonic fibroblast cell line purchased from American Type Culture Collection (Catalog No. CRL-1539). Cells were maintained in stocks of liquid nitrogen, thawed and grown in tissue culture flasks with RPMI 1640 (Gibco BRL, NY, USA) supplemented with 100 mL/L fetal bovine serum and 10 g/L penicillin/streptomycin at 37°C in a 50 mL/L CO₂ incubator. The cultured cells were trypsinized with fresh 2.5 g/L trypsin solution, trypsin was removed and the culture let sit at 37°C until the cells detached (about 5 min). Fresh media was added, aspirated and dispensed into new flasks. Subculture was done every 4-6 d.

MTT assay

A freshly prepared cell suspension was serially diluted in RPMI 1640 containing 100 mL/L FBS to give a cell density ranging 10⁹/L to 10¹¹/L counted by hemocytometer. After 24 h, the culture medium was replaced with a fresh medium containing hemoglobin, 5-FU, 5-DFUR or combination thereof. Six duplicate wells were set up in each sample. The cells not treated with the drugs served as control cells. After incubation time passed, 20 mL dimethylthiazol diphenyl tetrazolium bromide (MTT, 3 g/L) was added to each well and incubated at 37°C for 3 h. After removal of the medium, MTT stabilization solution (DMSO: ethanol = 1:1) was added, then shaken for 10 min until all crystal was dissolved. Then, optical density (OD) was detected in a microplate reader at 550 nm wavelength using an ELISA reader (EMAX ED927, Molecular Devices Inc., USA). The negative control well had no cells and was used as zero point of absorbance. Each assay was performed in triplicate. The following formula was used: cell proliferation inhibited (%) = [1-(A of the experimental samples/A of the control)] × 100%. Cell growth curve was completed using time as the abscissa and a value (mean ± SD) as the ordinate.

Measurement of ROS

Human hemoglobin was dissolved in RPMI 1640. HT 29

cells were incubated with 5-FU or 5'-fluoro-2'-deoxyuridine (5-FDUR) at 37°C in suspension culture at different concentrations 1 to 2 µg or for different incubation periods. These samples were processed for analysis of ROS by usual flowcytometric techniques. Briefly, cells were harvested, washed twice with PBS and resuspended in serum-free medium. They were incubated with 50 µmol/L 2',7'-dichlorofluorescein diacetate (Calbiochem, Meudon, France) for 2 h at 37°C and washed with ice-cold HEPES/saline and placed on ice. Fluorescence was measured by flowcytometry (Becton Dickinson, San Jose, USA). As a positive control, cells were separately treated with H₂O₂ and processed for ROS detection.

Immunoblot analysis

Harvested cell line extracts were homogenized with ice-cold lysis buffer (20 mmol/L HEPES pH 7.2, 150 mmol/L sodium chloride, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mg/L leupeptin, 10 mg/L aprotinin, 0.1 mmol/L DTT and 1 mmol/L phenylmethylsulfonyl fluoride). For the immunoblot analysis, extracted proteins of 10 µg were denatured by heating at 95°C for 10 min with Laemmli cooking buffer and separated on 120 g/L SDS polyacrylamide gel electrophoresis. The resolved protein bands were transferred onto a PVDF membrane (Amersham Biosciences, UK) and blocked non-specific binding site by immersing the membrane in 30 mL/L skim milk, 120 g/L Tween 20 in TBS for 2 h. The membrane was incubated with 1:2000 diluted primary antibody for peroxiredoxin I (Difco, USA) and superoxide dismutase 1 for 1 h at room temperature on an orbital shaker. The blotted membrane was then incubated using secondary antibody (anti-rabbit IgG, 1:3000) for 1 h at room temperature on an orbital shaker. Detection was performed with the ECL system.

Statistical analysis

Data shown in figures represent mean ± SEM. Unless otherwise stated, these means were calculated from the means of triplicate replicates obtained in at least three independent experiments. Statistical evaluation was performed with the Prism program version 6.0. Depending on sample size and type of experiment, repeated measures of ANOVA or one-way ANOVAs were used to determine the significance of the experimental variables.

RESULTS

Proliferation effect of hemoglobin

Cell growth was determined by MTT assay. HT-29 cell line, the main cell of colon cancer, and Lovo cell line and CCL-33Co cell line were objected to confirm the difference in proliferation dependant on the types of cells. It has been verified, as demonstrated in Figure 1, when injected with hemoglobin, proliferation in all three types of cell lines was much greater. We saw the time effects in HT-29 cell line. These effects began to display after 30 min of treatment and were most obvious after approximately 24 h (Figure 1A). After various adjustments of the concentration of the hemoglobin, the results showed that the higher

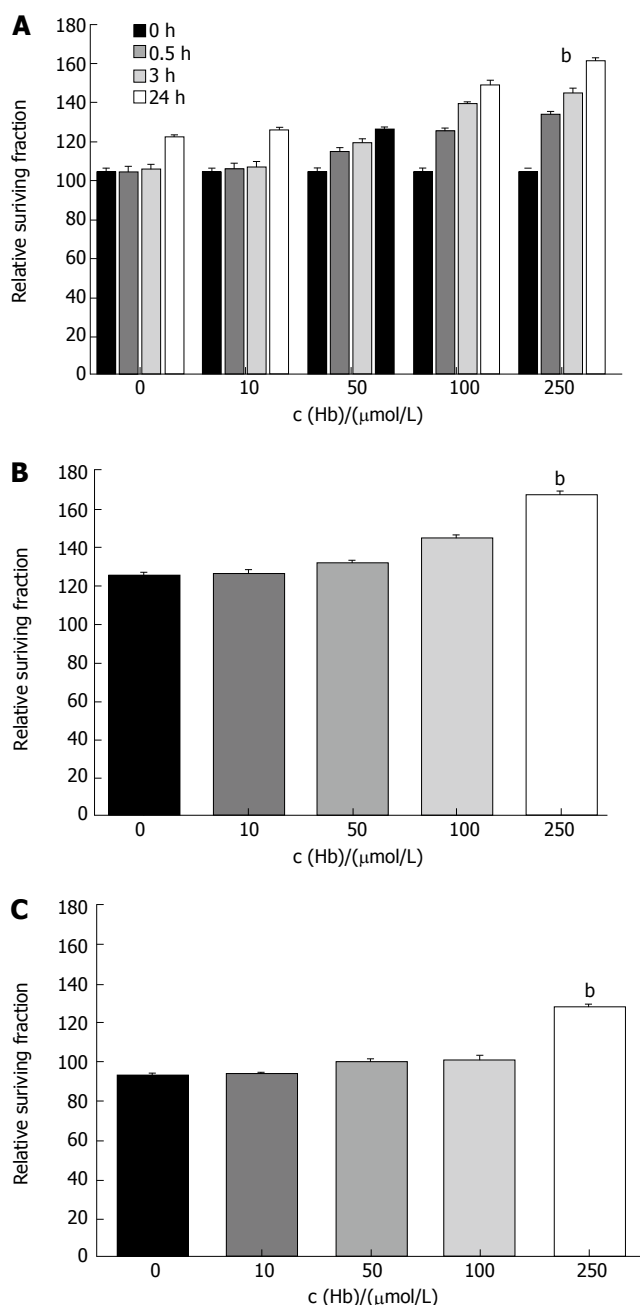


Figure 1 Time-course and dose-response effects of hemoglobin on apoptosis of colorectal cancer cell lines. **A:** HT-29 cell line. ^b*P* = 0.000 vs 0 μmol/L; **B:** Lovo cell line. ^b*P* = 0.000 vs 0 μmol/L; **C:** CCL-33Co cell line. ^b*P* = 0.000 vs 0 μmol/L.

the concentration, the greater the proliferation (Figure 1).

ROS production by hemoglobin

Flowcytometry using DEPC was conducted to measure the total quantity of ROS, which occurs during the administration of hemoglobin. As expected, the amount of ROS production showed a significant increase dependent on the concentration of hemoglobin (Figure 2), and ROS production increased subject to the amount of time following increment.

The restraint of proliferation by anti-cancer medicines

Two drugs (5-FU, an anti-cancer drug most commonly used in treatment of colon cancer, and 5-DFUR, the

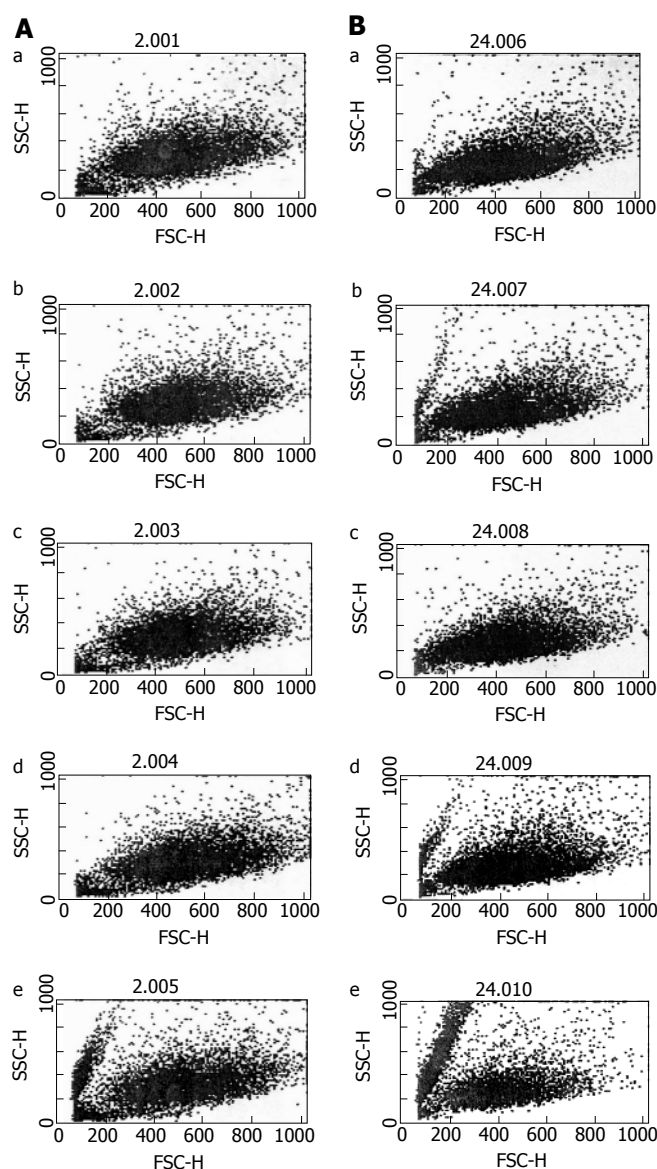


Figure 2 Flowcytometry of reactive oxygen species on HT-29 cells. **A:** After 3 h of hemoglobin administration; **B:** After 24 h of hemoglobin administration. a: Hb 0 μmol/L; b: Hb 10 μmol/L; c: Hb 50 μmol/L; d: Hb 100 μmol/L; e: Hb 250 μmol/L.

activating form of capecitabine which is presently used in metastatic colon cancer) were added to verify the constraint of proliferation effects. Twenty-four hours after drug application at varying concentrations, it was found that the higher the density, the greater was the constraint of proliferation in all three cell types (Figures 3 and 4). Also by flowcytometry, the anti-cancer medication decreases ROS production. Hence, it is believed that administration of anti-cancer drugs is effective in reducing ROS production (Figures 3D and 4D).

Influence of hemoglobin on anti-cancer agents

Twenty-four hours after simultaneously adding 5-FU, 5-DFUR and hemoglobin at various concentrations into three cell lines, results showed evidence of a weakening in the decreased proliferation as compared to using only anti-cancer drugs. Results were proportionate to the given densities of hemoglobin (Figures 5 and 6).

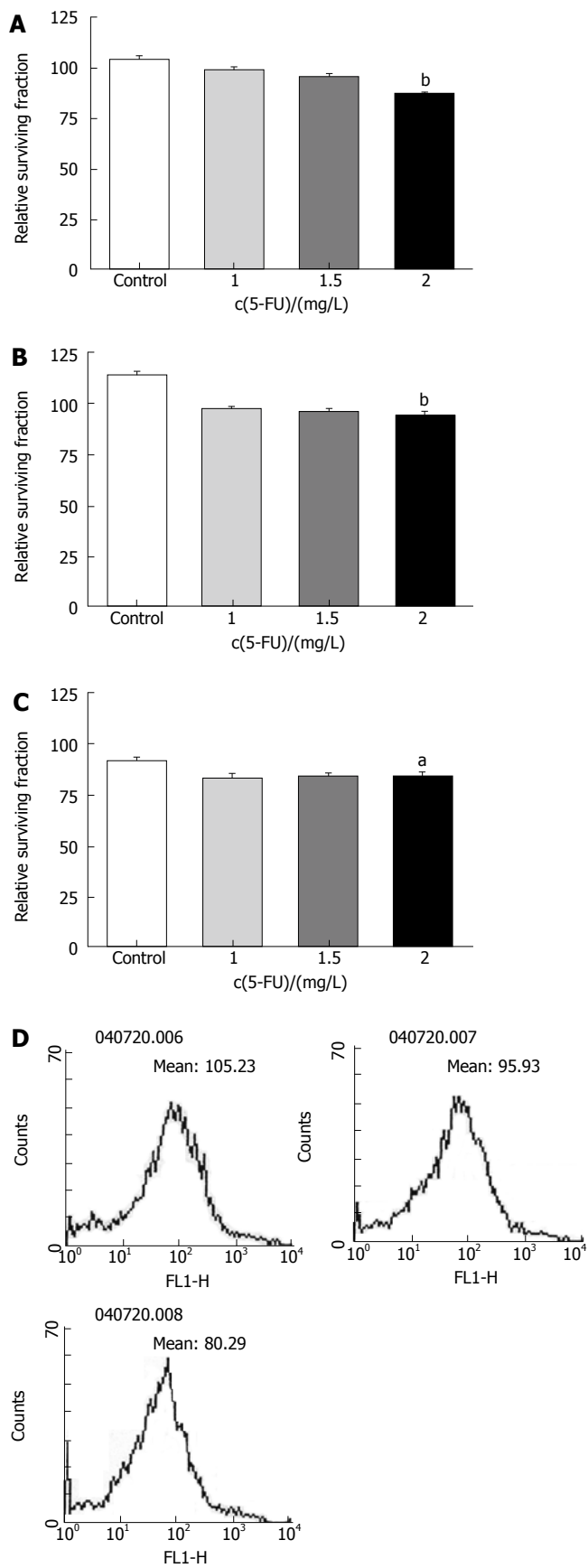


Figure 3 Relative surviving fraction of cell lines at 24 h after simultaneously adding each concentration of 5-fluorouracil. **A:** HT-29 cell line. ^b $P = 0.001$ vs Control; **B:** Lovo cell line. ^b $P = 0.003$ vs Control; **C:** CCL-33Co cell line. ^a $P = 0.039$ vs Control.

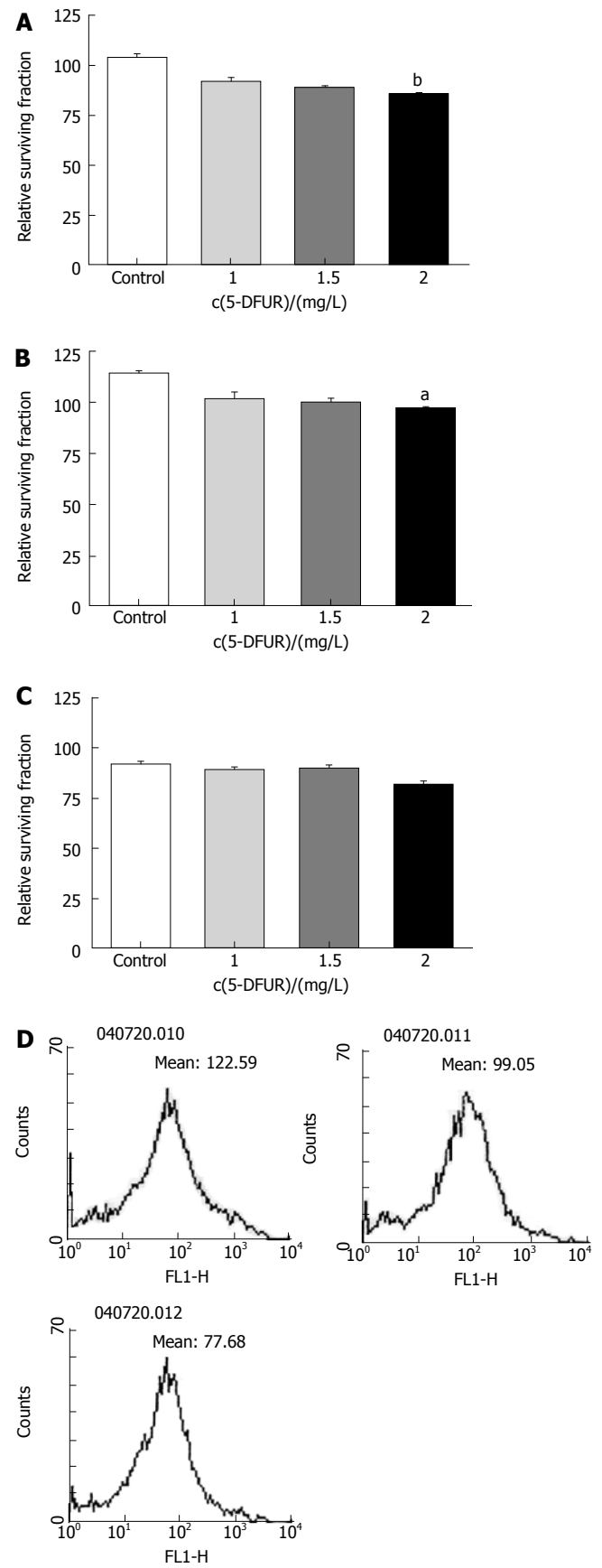


Figure 4 Relative surviving fraction of cell lines at 24 h after simultaneously adding each concentration of 5-DFUR. **A:** HT-29 cell line. ^b $P = 0.001$ vs Control; **B:** Lovo cell line. ^b $P = 0.003$ vs Control; **C:** CCL-33Co cell line. ^b $P = 0.010$ vs Control.

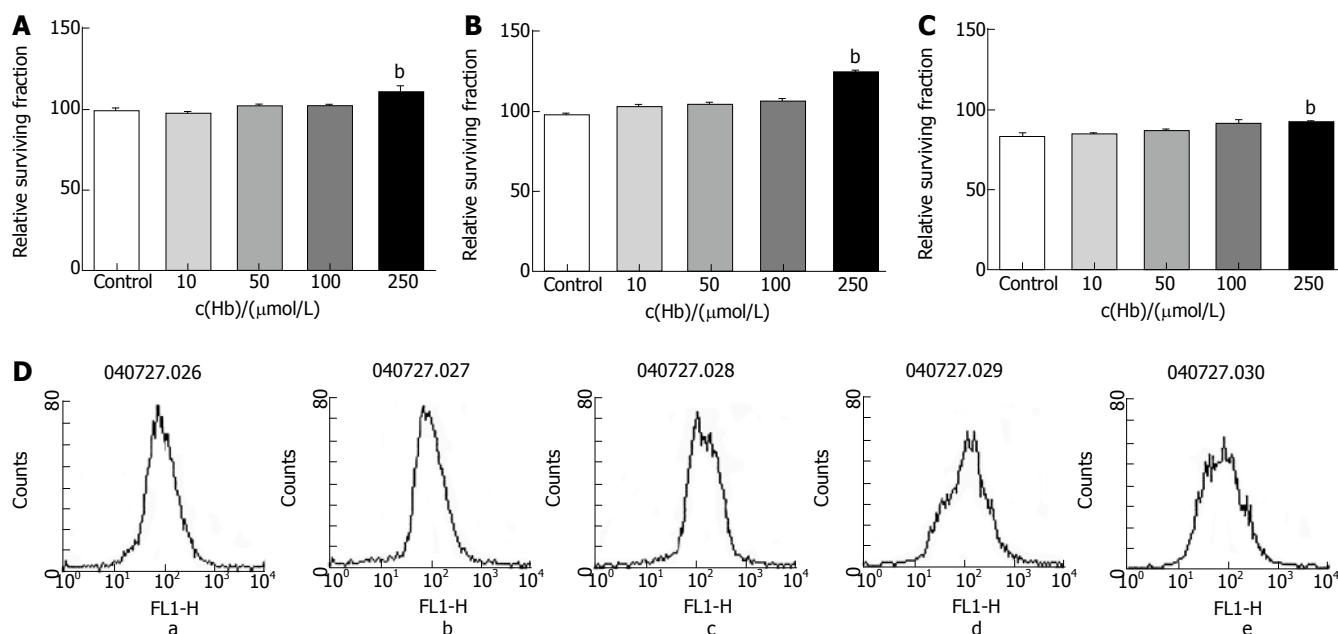


Figure 5 Relative surviving fraction of cell lines after adding 5-FU (1 mg/L) and each concentration of hemoglobin. **A:** HT-29 cell line. ^b*P* = 0.004 vs Control; **B:** Lovo cell line. ^b*P* = 0.000 vs Control; **C:** CCL-33Co cell line. ^b*P* = 0.005 vs Control; **D:** Flowcytometry of HT-29 cell line. a: Hb 0 μmol/L; b: Hb 10 μmol/L; c: Hb 50 μmol/L; d: Hb 100 μmol/L; e: Hb 250 μmol/L.

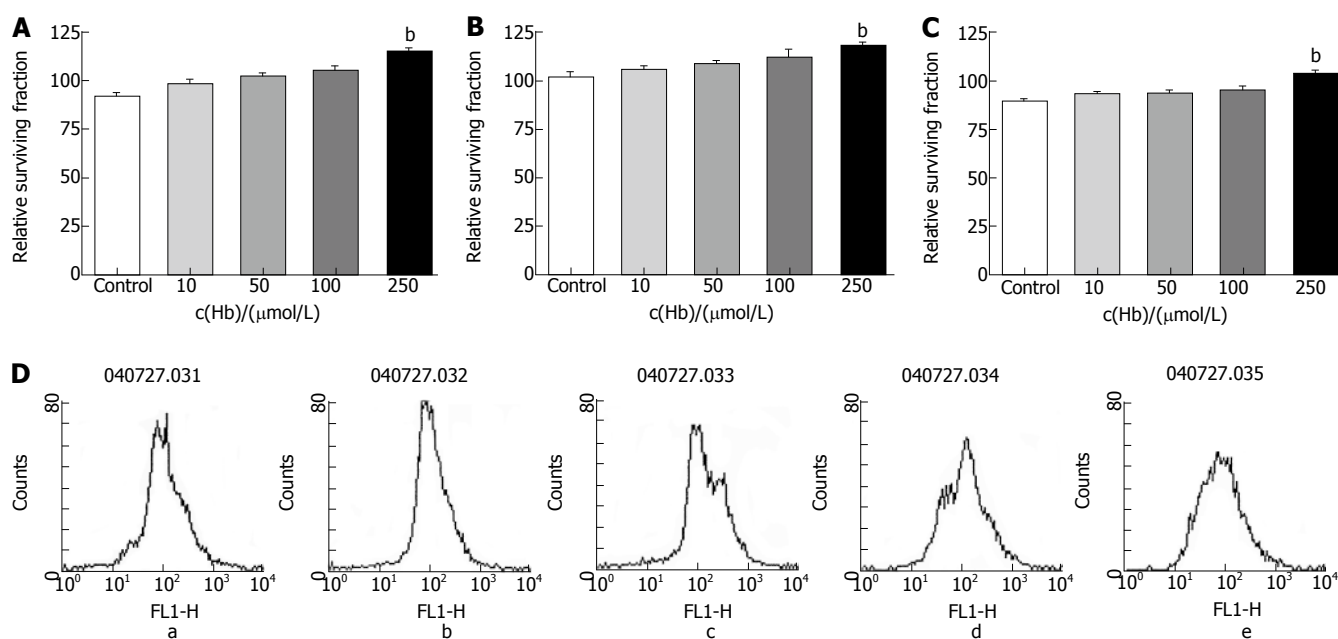


Figure 6 Relative surviving fraction of cell lines after adding 5-DFUR (1 mg/L) and each concentration of hemoglobin. **A:** HT-29 cell line. ^b*P* = 0.000 vs Control; **B:** Lovo cell line. ^b*P* = 0.006 vs Control; **C:** CCL-33Co cell line. ^b*P* = 0.000 vs Control; **D:** Flowcytometry of HT-29. a: Hb 0 μmol/L; b: Hb 10 μmol/L; c: Hb 50 μmol/L; d: Hb 100 μmol/L; e: Hb 250 μmol/L.

Expression of superoxide dismutase and peroxiredoxin

The occurrence of superoxide dismutase, the most important rate-limiting enzyme in ROS production *in vivo*, and peroxiredoxin, which is a producer of hydrogen peroxide (prominent type of ROS in cancer cells), was compared by the amount of hemoglobin added. Twenty four hours following treatment, the rate of occurrence of the two types of enzymes increased proportionately to the density of hemoglobin. However, the variation was only slight and less than expected (Figures 7 and 8).

DISCUSSION

High intake of meat is believed to be one of the main factors of high incidence of colon cancer among Western countries as compared with Asian countries. There are many reports dealing with which components and nutrients inside meat are considered most risky. There are plenty of reports about nutrients such as iron^[15], folate^[16], cholesterol^[17], calcium^[18], bile salt^[19], heterocyclic amines^[20] and vitamin D^[21] *etc.* related with various gastrointestinal malignancies. It is generally known that red meat has more

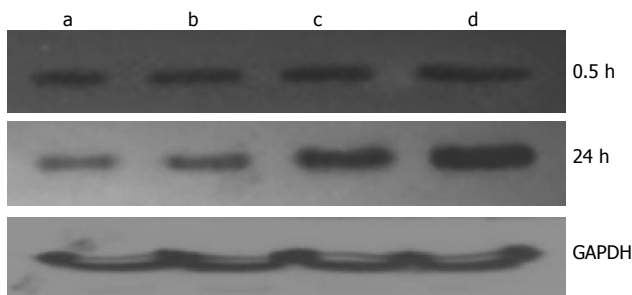


Figure 7 Change of Prx1 expression after administration of hemoglobin. a: Hb 0 $\mu\text{mol/L}$; b: Hb 10 $\mu\text{mol/L}$; c: Hb 100 $\mu\text{mol/L}$; d: Hb 250 $\mu\text{mol/L}$.

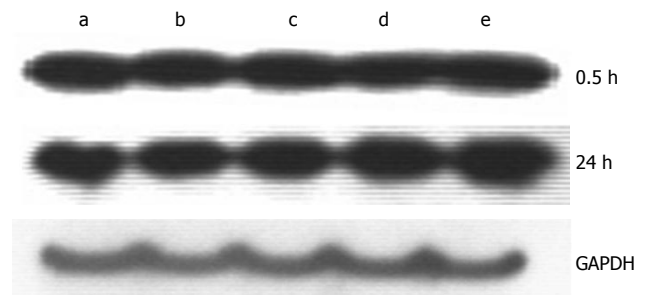


Figure 8 Change of SOD1 expression after administration of hemoglobin. a: Hb 0 $\mu\text{mol/L}$; b: Hb 10 $\mu\text{mol/L}$; c: Hb 50 $\mu\text{mol/L}$; d: Hb 100 $\mu\text{mol/L}$; e: Hb 250 $\mu\text{mol/L}$.

carcinogenic content than white meat. Due to the fact that some cancer patients believe red meat induces cancerous growths or encourages the recurrence thereof, they consume little to no red meats. Occasionally, malnutrition occurs as a result of extreme or reckless diet changes^[22]. However, conclusions about carcinogenic effects of red meat are not confirmed to date, especially the medical basis is insufficient. Therefore, *in vitro* research is required to effectively prove these carcinogenic effects.

The term 'red meat' refers mainly to mammal flesh such as beef, pork and lamb, *etc.* as with most edible meats, whereas the term 'white meat' refers mainly to fowl. The ruddy coloring of red meat reflects the density of myoglobin that is found in muscle tissue and some roles of hemoglobin are also included. Hemoglobin is a tetramer consisting of two alpha chains and two beta chains. Since each unit can combine with oxygen wherein iron is present, 1 hemoglobin can carry 4 oxygen molecules. The main function of hemoglobin is to carry oxygen, in addition to the storage of iron and the place providing globin. This type of function occurs only *in vivo* and exists within the bloodstream. When hemoglobin is inducted into the digestive tract, it is dissolved by digestive enzymes as are other proteins and absorbed as a protein and other nutrients. There are a few reports on the carcinogenic effects of heme or hemoglobin among the components of meat^[10, 23-27]. Sesink *et al.*^[28] stated that dietary heme effects on colon epithelial hyperproliferation are hindered by calcium. They administered pure hemin in a meal of an F344 female rat. They announced that the resulting effect of a low calcium and heme containing diet increases colon wall aberrant crypt foci (ACF). Into an experimental model by Pierre *et al.*^[10] heme and hemoglobin were injected and the size and number of ACF were compared and measured with fecal thiobarbituric acid reactive substances (TBAR). In the case of hemoglobin, the number of ACF and the amount of TBAR increased. From these results, hemoglobin was announced as a potent promoter of colorectal carcinogenesis. Gleit *et al.*^[14] observed the increase of DNA strand break after the iron overload injected Fe-NTA (ferric-nitrilotriacetate) which was synthesized from the ferric nitrate and nitrilotriacetic acid into HT29 clone 19A cell line. Also, it was reported that when peroxide was added, more DNA strand breaks occurred with increasing peroxide concentrations. With these findings, iron content within a hemoglobin containing diet increased the DNA genotoxicity. We proceeded with this study under the

assumption that hemoglobin components of dietary red meat affects cell proliferation of the cancerous and normal colonic cells via production of ROS. As expected, the results of this study confirmed the leading effect of hemoglobin. By the amount of hemoglobin dealt with, not only the cellular proliferation increased but also the amount of ROS production increased supporting predictions thereof. The proliferation effect of hemoglobin on normal colonic fibroblast was noted similarly in cancer cell lines. It is assumed that it presents the effect that exposure of hemoglobin leads to normal colon proliferation as well and produces ROS, which forms much more DNA breaks and also helps in becoming susceptible to other carcinogenic stimuli. Enzymes related with ROS production such as peroxiredoxin 1^[29] and superoxide dismutase 1^[30] also increased after hemoglobin application but the effect was minimal compared with expectations. These results suggested that production of enzymes was not a major factor in the process of colon cancer cell proliferation via ROS production.

5-FU and 5-DFUR are major chemotherapeutic agents in the treatment of colorectal cancer^[31,32]. We used these drugs to identify adverse effects of hemoglobin on chemotherapy. Cellular proliferation was decreased 12 h after adding the 5-FU or 5-DFUR and ROS production was decreased as expected. The results with 5-FU was similar with that of 5-DFUR. These reactions were affected by administration of hemoglobin in a concentration-dependent manner in all three cell lines. ROS production was also decreased with hemoglobin. It is interpreted that hemoglobin administration restricted the cytotoxic effect of anti-cancer drugs in colon cancer cells and normal colonic fibroblasts.

In conclusion, hemoglobin inside red meat has a promoting effect on cellular proliferation in cancer cells and in normal colonic fibroblast cells by release of ROS. Furthermore, this phenomenon reduces the cytotoxicity of anticancer drugs, such as 5-FU and 5-DFUR, to colon cancer cells, which could be an adverse factor during chemotherapy in a clinical setting.

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Promoter hypomethylation and reactivation of *MAGE-A1* and *MAGE-A3* genes in colorectal cancer cell lines and cancer tissues

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Abstract

AIM: To verify the expression and methylation status of the *MAGE-A1* and *MAGE-A3* genes in colorectal cancer tissues and cancer cell lines.

METHODS: We evaluated promoter demethylation status of the *MAGE-A1* and *MAGE-A3* genes by RT-PCR analysis and methylation-specific PCR (MS-PCR), as well as sequencing analysis, after sodium bisulfite modification in 32 colorectal cancer cell lines and 87 cancer tissues.

RESULTS: Of the 32 cell lines, *MAGE-A1* and *MAGE-A3* expressions were observed in 59% and 66%, respectively. Subsequent to sodium bisulfite modification and MS-PCR analysis, the promoter hypomethylation of *MAGE-A1* and *MAGE-A3* was confirmed in both at 81% each. Promoter hypomethylation of *MAGE-A1* and *MAGE-A3* in colorectal cancer tissues was observed in 43% and 77%, respectively. Hypomethylation of *MAGE-A1* and *MAGE-A3* genes in corresponding normal tissues were observed in 2% and 6%, respectively.

CONCLUSION: The promoter hypomethylation of *MAGE* genes up-regulates its expression in colorectal carcinomas as well as in gastric cancers and might play a significant role in the development and progression of human colorectal carcinomas.

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Key words: *MAGE-A1*; *MAGE-A3*; Promoter; Hypomethylation; Colorectal cancer

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INTRODUCTION

Human tumors often display changes in DNA methylation, which include both genome-wide hypomethylation and site-specific hypermethylation. Global hypomethylation and CpG island hypermethylation have been recognized as important contributors to the development of carcinogenesis in humans. Hypermethylation of promoter CpG islands is the signature of transcriptional silencing of their downstream genes, including *RB*, *p16*, *VHL*, *BRCA1*, *E-cadherin*, *APC*, *bMLH1*, *FHIT*, *COX2*, and *CDX1* in various human cancers; and is as effective as inactivation by gene mutation or deletion^[1-6]. Global DNA hypomethylation has been implicated in the activation of oncogenes such as *c-myc*, *k-ras*, and it may also contribute to tumor progression by the induction of genome instability^[7,8].

The *MAGE* family of genes belongs to a group of germ line-specific genes that are activated in different types of tumors. This family of genes was reported to direct the expression of a tumor-specific antigen that was recognized in a melanoma cell by cytolytic T lymphocytes^[9]. The *MAGE-A1* gene has a CpG-rich promoter, which, unlike classical CpG-rich promoters, is methylated in all normal somatic tissues, except for the placenta and testis. In contrast, the promoter region of *MAGE-A1* is completely unmethylated in testicular germ cells and in tumor cells that express the gene^[10]. Demethylation, and therefore, activation of *MAGE-A1* in tumors appears to be a consequence of the genome-wide demethylation process, since the expression of this gene in tumor cells correlates with a decreased level of overall DNA methylation^[11]. A correlation between *MAGE-A1* and *MAGE-A3* expression and genome-wide hypomethylation has been observed in some types of carcinomas^[12,13]. The human *MAGE-A1* and *MAGE-A3* genes, which are located on chromosome X, are expressed in 29% and 66%, respectively, of human gastric cancer cells due to the hypomethylation of the promoter region^[12]. However, it is unknown if this relationship is present in colorectal carcinomas.

In this study, we investigated the promoter methylation

status of *MAGE-A1* and *MAGE-A3* genes. A total of 32 colorectal cancer cell lines were tested for hypomethylation of the *MAGE-A1* and *MAGE-A3* genes promoter. In addition, we screened the methylation status of the *MAGE-A1* and *MAGE-A3* genes promoter in 87 paired colorectal cancers and normal mucosal tissue samples.

MATERIALS AND METHODS

Cell cultures

A total of 32 colorectal cancer cell lines (Table 1) and 2 gastric cancer cell lines (SNU-1 and SNU-5) were obtained from either the Korean Cell Line Bank (KCLB; Seoul, Korea) or the American Type Culture Collection (ATCC; Manassas, VA, USA). Sixteen SNU-colorectal cancer cell lines were established and were reported upon previously by this laboratory^[14,15]. SNU-1 and SNU-5 gastric carcinoma cell lines were used as methylation positive (SNU-1) and negative (SNU-5) controls for *MAGE* gene expression^[12]. All the cell lines were maintained in RPMI1640, which was supplemented with 10% FBS, 100 kU/L penicillin, and 0.1 g/L streptomycin. The cultures were maintained in humidified incubators at 37°C in a 5% CO₂ and 95% ambient air atmosphere.

Nucleic acid isolation and cDNA synthesis from the cell lines

Genomic DNA and total RNA were isolated from washed-cell pellets. Total genomic DNA was extracted in accordance with the standard SDS-proteinase K procedure; and total cellular RNA was extracted based on the manufacturer's instructions (Intron Biotechnology; Seoul, Korea). For cDNA synthesis, 2 µg of total RNA was reverse transcribed with a random hexamer, dNTPs, and 1 µL (200 U) of SuperscriptTM II reverse transcriptase (Life Technologies; Gaithersburg, MD, USA) in a final volume of 20 µL for 1 h and 15 min at 42°C after a 10-min denaturation at 70°C. Eighty microliters of distilled water were added subsequent to the reverse-transcription reaction.

Expression of *MAGE-A1* and *MAGE-A3* genes

For mRNA expression analysis, the cDNA was amplified in 25 µL of a PCR reaction mix with 1 µL of the reverse-transcription reaction, the primers and 0.5 U of Taq DNA polymerase. The PCR conditions consisted of 10 min at 94°C for the initial denaturation, followed by 35 cycles of 94°C for 30 s, 54°C for 60 s, and 72°C for 60 s, and a final elongation of 7 min at 72°C. The primer sequences are as follows. *MAGE-A1* cDNA was amplified by PCR with MG1 RT primers; MG1 RT sense, 5'-TGTGGG CAGGAGCTGGGCAA-3', MG1 RT antisense, 5'-GCCGAAGGAACCTGACCCAG-3'. For the *MAGE-A3* cDNA, the MG3 RT primers were used; MG3 RT sense, 5'-AAGCCGGCCAGGCTCGGT-3', MG3 RT antisense, 5'-GCTGGGCAATGGAGACCCAC-3'. PCR amplification was performed in a programmable thermal cycler (PCR System 9700, Applied Biosystems; Foster City, CA, USA). Primers for β -actin were used to confirm RNA integrity. Both *MAGE-A1* and *MAGE-A3* and β -actin RT-PCR reactions used the same cDNA synthesis. The

amplified DNA fragments were fractionated in 2% agarose gel and stained with ethidium bromide.

Tissue sample collection and DNA extraction

A total of 87 paired tumor and normal mucosal tissue samples were obtained from 87 patients, who had primary colorectal adenocarcinoma. The normal mucosal tissue specimens were collected from each patient 10 cm or more away from the tumor areas. Approximately 2 g of the surgically removed tissues were frozen immediately and then stored in liquid nitrogen. The remaining sections of the samples were fixed with formalin and used for further histological examination in order to confirm the diagnosis postoperatively. Genomic DNA was isolated from the frozen-tissue biopsies with the standard SDS-proteinase K procedure.

Methylation specific PCR

With respect to the MS-PCR, the sodium bisulfite modification of genomic DNA was performed as reported previously^[16]. A total of 2 µg of genomic DNA obtained from colorectal cancer cell lines, was denatured with NaOH and hydroquinone. Then, 3 mmol/L sodium bisulfite was added and the mixture was incubated at 55°C for 16 h. Following the bisulfite modification, the DNA was purified with a Wizard DNA purification system (Promega; Madison, WI, USA), ethanol precipitated, dried, and resuspended in 100 µL distilled water. The PCR was performed using the PCR primers that were described previously^[12]. The amplified DNA fragments were fractionated in 2% agarose gel that was stained with ethidium bromide and visualized under UV light.

5-aza-2'-deoxycytidine treatment and RT-PCR

For 5-aza-2'-deoxycytidine treatment, the cells were seeded in two 2×10^5 cells/75 cm² culture flasks on d 0. The cells were treated with and without 1-5 µmol/L of 5-aza-2'-deoxycytidine (Sigma Chemical Co.) for 24 h on d 2 and 5, and the medium was changed 24 h after addition of 5-aza-2'-deoxycytidine. The cells were harvested on d 8 for the analysis of the *MAGEs* expression. Subsequently, the RNA was prepared, and RT-PCR was performed to detect *MAGE-A1* and *MAGE-A3* expression with the *MAGE-A1* and *MAGE-A3* RT-PCR primers as described above.

RESULTS

Expression of *MAGE-A1* and *MAGE-A3* in colorectal cancer cell lines

Expression of *MAGE-A1* and *MAGE-A3* mRNA in 32 colorectal cancer cell lines was analyzed by RT-PCR, and *MAGE-A1* and *MAGE-A3* expressions were observed in 19 (59%) and 21 (66%) of the cell lines, respectively (Figure 1 and Table 1). PCR for β -actin confirmed the integrity of the RNA.

Clinico-pathological features

Of the 87 colorectal carcinomas, 57 (66%) were obtained from the proximal colon (cecum to splenic flexure), and 30 (34%) from the distal colorectum (splenic flexure to

Table 1 Methylation status of the promoter region of *MAGE-A1* and *MAGE-A3* genes in 32 colorectal cancer cell lines

Cell line		<i>MAGEs</i> expression				MS-PCR			
		<i>MAGE-A1</i>		<i>MAGE-A3</i>		<i>MAGE-A1</i>		<i>MAGE-A3</i>	
		-5-aza/ +5-aza		-5-aza/ +5-aza		M/U		M/U	
1	SNU-61	-	++	±	++	+	+	-	+
2	SNU-81	-	++	-	+++	+	-	+	-
3	SNU-175	++	++	+++	+++	+	+	+	+
4	SNU-283	+++	NT	+++	NT	+	+	+	-
5	SNU-407	+++	+	+++	++	+	-	+	+
6	SNU-503	++	NT	-	NT	+	+	+	+
7	SNU-769A	+++	++	+++	+++	-	+	-	+
8	SNU-769B	+++	+++	+++	+++	-	+	-	+
9	SNU-1033	++	+	++	+++	+	+	+	+
10	SNU-1040	-	-	±	++	+	-	+	-
11	SNU-1047	+++	++	++	++	+	-	-	+
12	SNU-1197	++	+++	++	+++	+	+	+	+
13	SNU-C1	±	NT	++	NT	+	+	-	+
14	SNU-C2A	++	NT	+++	NT	+	+	+	+
15	SNU-C4	-	NT	-	NT	+	-	+	+
16	SNU-C5	±	++	-	+++	+	+	+	-
17	Caco-2	-	++	++	+++	+	+	+	+
18	COLO201	-	++	-	+++	-	+	-	+
19	COLO205	-	-	-	++	+	+	+	+
20	COLO320	+++	NT	+++	NT	+	+	-	+
21	DLD-1	-	+++	-	+++	+	+	+	+
22	HCT-8	-	++	-	+++	+	-	+	-
23	HCT-15	-	++	-	+++	+	+	+	+
24	HCT-116	+++	NT	+++	NT	+	+	+	+
25	HT-29	+++	++	+++	+++	+	+	+	+
26	Lovo	+++	+++	+++	+++	+	+	+	+
27	LS174T	+++	NT	+++	NT	+	+	-	+
28	NCI-H716	+++	+++	+++	+++	+	+	-	+
29	SW403	+++	++	+++	+++	+	+	+	+
30	SW480	+++	NT	+++	NT	+	+	+	-
31	SW1116	-	NT	++	NT	+	+	-	+
32	WiDR	++	+++	+++	+++	+	+	-	+

5-aza: 5-aza-2'-deoxycytidine; NT: Not tested; M: The amplified product with primers recognizing methylated sequence; U: The amplified product with primers recognizing unmethylated sequence; +: Amplified product; -: Not amplified product.

rectum). Randomly selected patients aged 16-81 years, including 55 males and 32 females. Of the 32 colorectal cancer cell lines, 7 originated from the proximal colon and 8 from the distal colorectum. The origin of the remaining 17 colorectal cancer cell lines was unknown.

Analysis of *MAGE-A1* and *MAGE-A3* methylation by MS-PCR

By using primers for unmethylated *MAGE-A1* DNA amplification on bisulfite modified DNA, amplified DNA fragments were observed in 26 (81%) cell lines (SNU-61, SNU-175, SNU-283, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1197A, SNU-C1, SNU-C2A, SNU-C5, Caco2, COLO201, COLO205, COLO320, DLD-1, HCT-15, HCT-116, HT-29, Lovo, LS174T, NCI-H716, SW403, SW480, SW1116, and WiDR) (Figure 2 and Table 1). And by using primers for unmethylated *MAGE-A3* DNA amplification on bisulfite modified DNA, amplified DNA fragments were found also in 26 (81%) cell lines (SNU-61, SNU-175, SNU-407, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1047, SNU-

1197A, SNU-C1, SNU-C2A, SNU-C4, Caco2, COLO201, COLO205, COLO320, DLD-1, HCT-15, HCT-116, HT-29, Lovo, LS174T, NCI-H716, SW403, SW1116, and WiDR) (Figure 2 and Table 1). By using primers for amplification of unmethylated or methylated DNA, amplified DNA fragments were found in all 32 cell lines. *MAGE-A1* unmethylated DNA products were observed in 37 out of 87 tumor tissue samples (43%; Figure 3). In the normal tissue samples, the methylated DNA was amplified in all 87 samples. However, the unmethylated DNA was amplified in 2 normal tissues (2%). *MAGE-A3* unmethylated DNA products were observed in 67 out of 87 tumor tissue samples (77%; Figure 3). In the normal tissue samples, the methylated DNA was amplified in all 87 samples. However, the unmethylated DNA was amplified in 5 normal tissues (6%).

Reexpression of *MAGE-A1* and *MAGE-A3* after treatment with 5-aza-2'-deoxycytidine

We investigated whether *MAGE-A1* mRNA was re-expressed after 5-aza-2'-deoxycytidine treatment in 22 cell

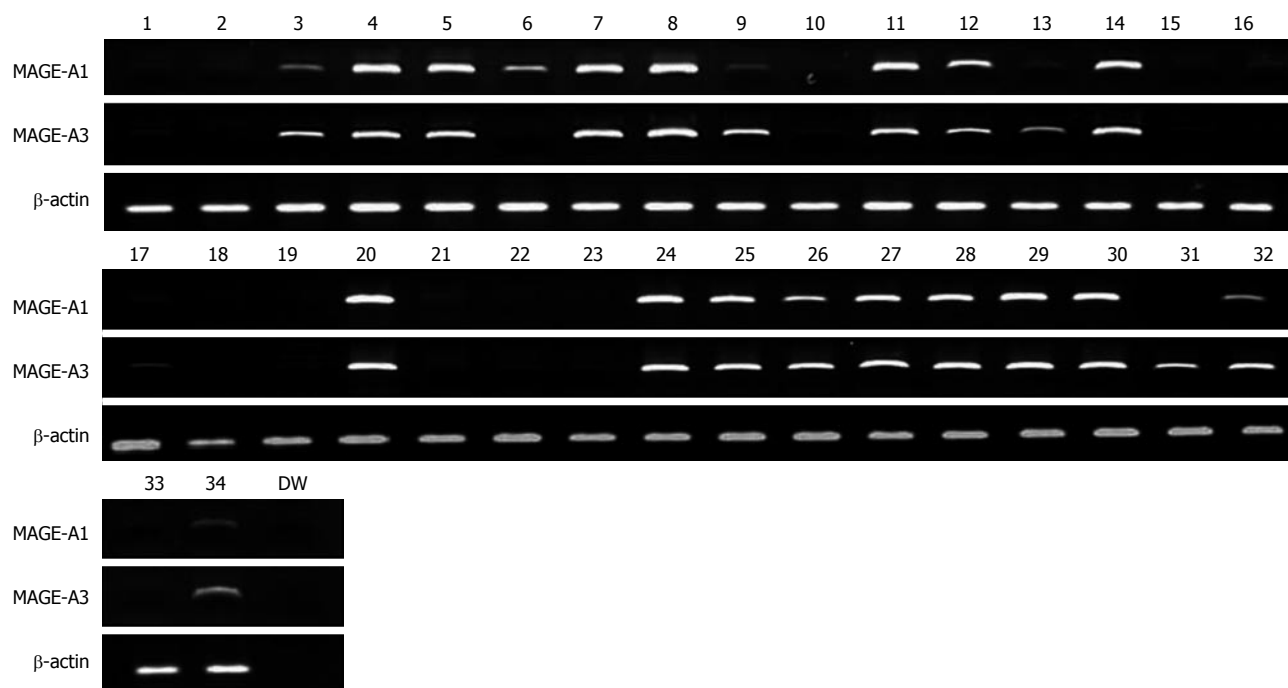


Figure 1 RT-PCR analysis of the *MAGE-A1* and *MAGE-A3* genes in 32 colorectal cancer cell lines. β -actin was amplified as an internal control. The *MAGE-A1* gene was significantly expressed in 19 colorectal cancer cell lines (Lanes, 3, 4, 5, 6, 7, 8, 9, 11, 12, 14, 20, 24, 25, 26, 27, 28, 29, 30 and 32). The *MAGE-A3* gene was expressed in 21 colorectal cancer cell lines (Lanes, 3, 4, 5, 7, 8, 9, 11, 12, 13, 14, 17, 20, 24, 25, 26, 27, 28, 29, 30, 31 and 32). Lane numbers 1 to 34 show cell lines SNU-61, SNU-81, SNU-175, SNU-283, SNU-407, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1040, SNU-1047, SNU-1197, SNU-C1, SNU-C2A, SNU-C4, SNU-C5, Caco-2, COLO201, COLO205, COLO320, DLD1, HCT-8, HCT-15, HCT-116, HT-29, LOVO, LS174T, NCI-H716, SW403, SW480, SW1116, WiDr, SNU-1, and SNU-5 respectively.

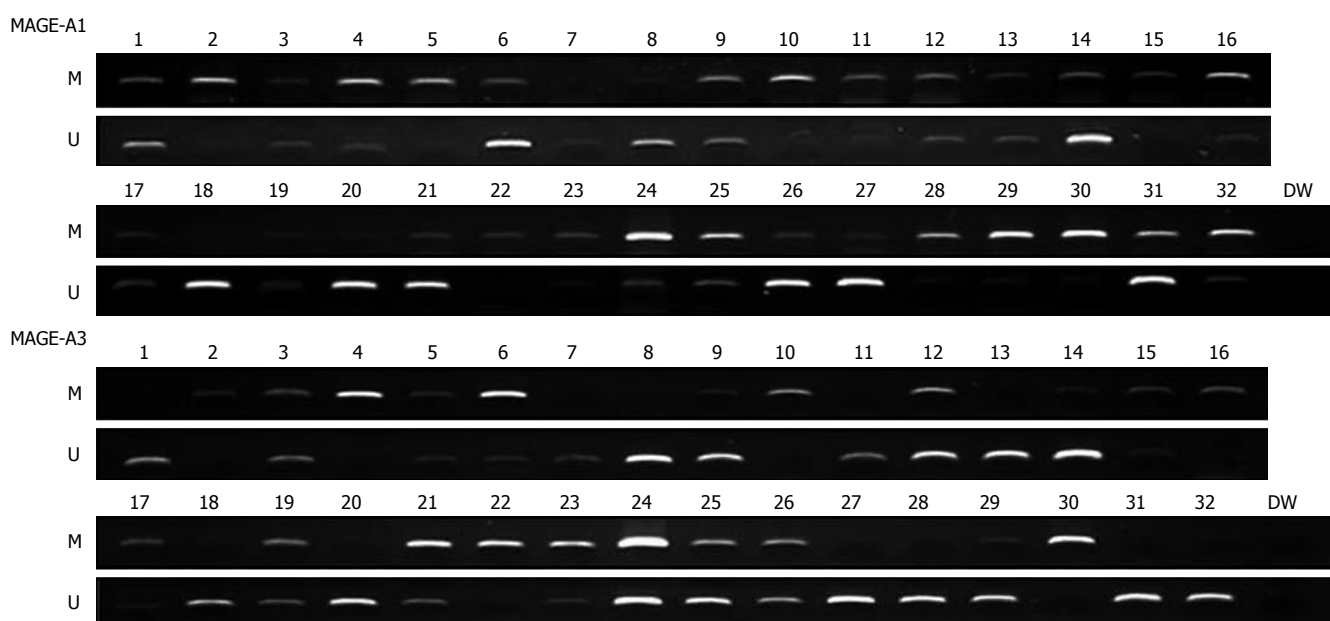


Figure 2 Methylation analysis of the *MAGE-A1* and *MAGE-A3* genes in colorectal cancer cell lines. The promoter region of the *MAGE-A1* gene was unmethylated in 26 cell lines (SNU-61, SNU-175, SNU-283, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1197A, SNU-C1, SNU-C2A, SNU-C5, Caco2, COLO201, COLO205, COLO320, DLD-1, HCT-15, HCT-116, HT-29, Lovo, LS174T, NCI-H716, SW403, SW1116, and WiDR cell lines). Unmethylated *MAGE-A3* DNA amplifications were found in 26 cell lines (SNU-61, SNU-175, SNU-407, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1047, SNU-1197A, SNU-C1, SNU-C2A, SNU-C4, Caco2, COLO201, COLO205, COLO320, DLD-1, HCT-15, HCT-116, HT-29, Lovo, LS174T, NCI-H716, SW403, SW1116, and WiDR cell lines). Lane M denotes product amplified by primers recognizing a methylated sequence and Lane U denotes the product amplified by primers recognizing an unmethylated sequence, respectively.

lines, including 10 cell lines (SNU-61, SNU-81, SNU-1040, SNU-C5, Caco-2, COLO201, COLO205, DLD1, HCT-8, and HCT-15) that did not express *MAGE-A1* mRNA. After an RT-PCR analysis, we observed that all of the *MAGE-A1* mRNAs were re-expressed, except for the SNU-1040 and COLO205 cell lines (Table 1).

This suggested that the inactivation of *MAGE-A1* expression was caused by another mechanism. Further, we investigated whether *MAGE-A3* mRNA was re-expressed after 5-aza-2'-deoxycytidine treatment in 22 cell lines, including 9 cell lines (SNU-61, SNU-81, SNU-1040, SNU-C5, COLO201, COLO205, DLD1, HCT-8, and

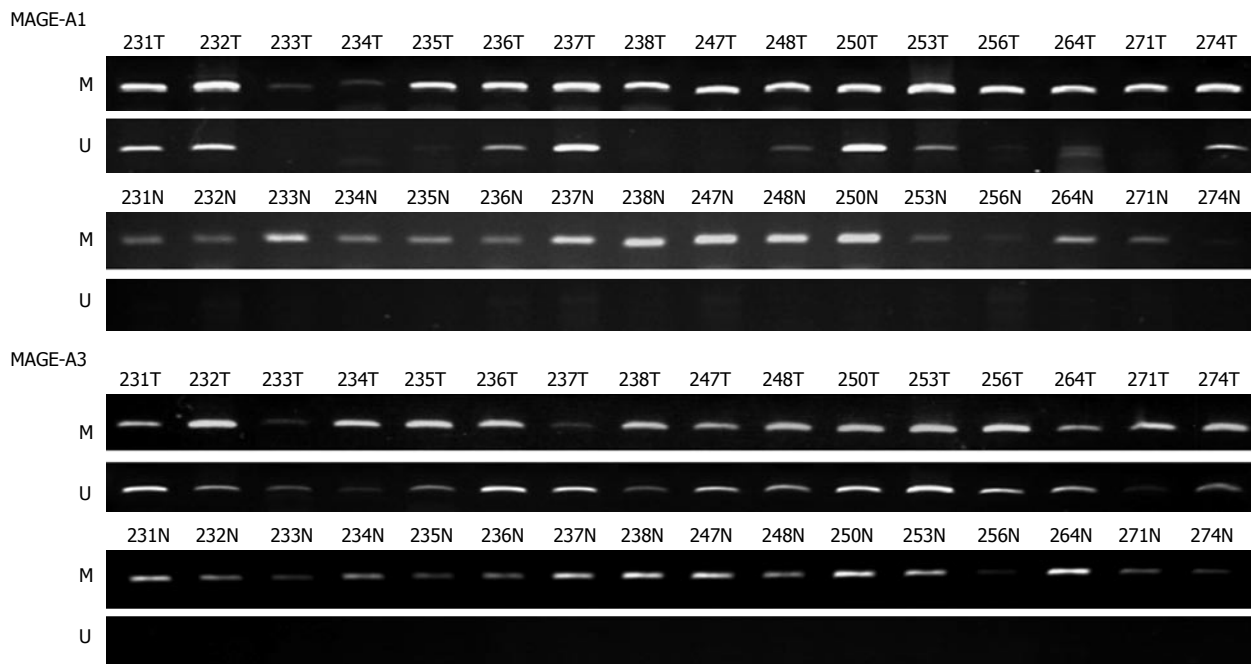


Figure 3 Methylation analysis of the *MAGE-A1* and *MAGE-A3* genes in colorectal cancer tissues and corresponding normal tissues. Methylation-specific PCR product amplified by primers recognizing methylated and unmethylated sequence. The promoter region of the *MAGE-A1* and *MAGE-A3* genes was unmethylated in colorectal cancer tissues, not normal tissues. Numbers represent each colorectal tissue and T denotes colorectal tumor tissues and N denotes corresponding normal tissues.

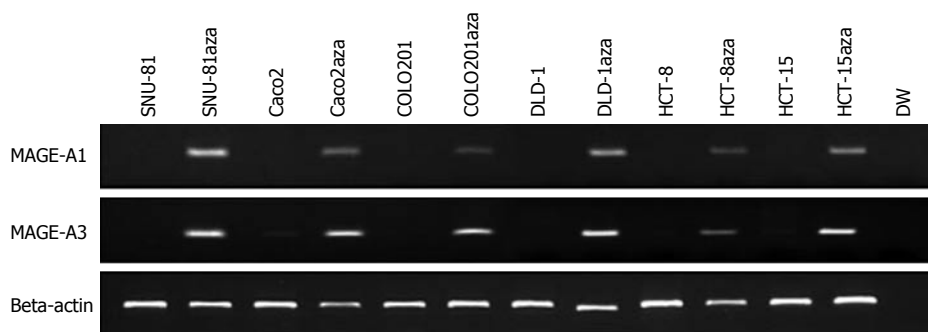


Figure 4 RT-PCR analysis after treatment with 5-aza-2'-deoxycytidine. The *MAGE-A1* and *MAGE-A3* genes were reactivated.

HCT-15) that did not express *MAGE-A3* mRNA. After an RT-PCR analysis, we observed that all of the *MAGE-A3* mRNAs were re-expressed (Table 1 and Figure 4).

DISCUSSION

Genome-wide hypomethylation and site-specific hypermethylation are common features of cancer cells. DNA hypomethylation in cancer cells is accompanied by the activation of germ line-specific genes, such as the *MAGE-A1* gene, the repression of which, in normal somatic tissues, is dependent upon DNA methylation^[17]. Recent studies have reported the presence of very high *MAGE-A1* and *MAGE-A3* expressions in colorectal carcinomas^[18,19]. Although previous reports have shown the expression of *MAGE* genes, the mechanism of *MAGE* genes expression in colorectal carcinomas was unclear. This led us to question whether it could be associated with decreased genomic methylation. It has been reported that *MAGE-A1* and *MAGE-A3* expression was related to gene hypomethylation in gastric carcinoma, hepatocarcinoma, and melanoma^[12,20,21]. However, such a relationship has still not been confirmed in colorectal

carcinoma. Accordingly, we analyzed the methylation status of the promoter region on the *MAGE-A1* and *MAGE-A3* genes in 32 colorectal cancer cell lines and estimated its association with *MAGE-A1* and *MAGE-A3* mRNA expression. We first examined the expression pattern of the *MAGE-A1* and *MAGE-A3* genes in these cell lines by an RT-PCR and observed that *MAGE-A1* and *MAGE-A3* were over-expressed significantly in 19 (59%) and 21 (66%) cell lines, respectively. This expression ratio of *MAGE-A1* and *MAGE-A3*, obtained by an RT-PCR in colorectal cancer cell lines, is similar to that observed in gastric cancer cell lines^[12]. On the other hand, previous reports have revealed a much lower expression of *MAGE* in colorectal carcinomas ranging between 5%-39%^[18,19,22]. In the literature, the expression of the *MAGE* genes was studied in colorectal carcinoma tissues; however, we have tested that in cancer cell lines. We assume that the major discrepancy of expression rates of *MAGE* genes between other reports and our findings might result from this. Since RNA or DNA was extracted from surgically removed frozen tissue biopsies, the tumor tissues may have been contaminated with normal stromal cells, therefore, masking the true levels of hypomethylation or expression

Table 2 Summary of expressions, promoter methylation status and clinical associations of the *MAGE-A1* and *MAGE-A3* genes in 32 colorectal cancer cell lines and 87 colorectal cancer tissues

	% of expression			% of hypomethylation		
	<i>MAGE-A1</i>	<i>MAGE-A3</i>		<i>MAGE-A1</i>	<i>MAGE-A3</i>	
Cancer cell lines	59	66		81	81	
Cancer tissues	NT ¹	NT		43	77	
Normal tissues ²	NT	NT		2	6	
	<i>MAGE-A1</i> hypomethylation			<i>MAGE-A3</i> hypomethylation		
	+	-	<i>P</i>	+	-	<i>P</i>
Location						
Proximal	14 (31.1%)	31(68.9%)		34 (75.6%)	11 (24.4%)	
Distal	17 (56.7%)	13(43.3%)	0.028	26 (86.7%)	4 (13.3%)	0.239
Sex						
Male	17 (30.9%)	38(69.1%)		38 (69.1%)	17 (30.9%)	
Female	20 (62.5%)	12(18.4%)	0.004	29 (90.6%)	3 (9.4%)	0.021

¹Not tested; ²Corresponding normal tissues of cancer tissues; +: Represent hypomethylation of *MAGE* genes; -: Represent no hypomethylation of *MAGE* genes.

of the *MAGE* genes in cancer tissues^[6]. For further analysis of the methylation status of the *MAGE* genes in colorectal cancer tissues, laser capture microdissection techniques would allow more precise isolation of cancer cells and normal cells. It has already been reported that cancer cell lines have much higher levels of CpG island hypermethylation than corresponding malignant tissues, which may explain our lower incidence of hypomethylation in tissues *versus* cell lines. Moreover, cancer cells might be clonally selected with growth advantages over cancer cell lines. However, cancer cell lines often preserve hypermethylation or hypomethylation from the tumors they originate, thus they are indeed useful tools to study methylation status.

We analyzed promoter unmethylation of the *MAGE-A1* and *MAGE-A3* genes with a methylation-specific PCR after sodium-bisulfite modification and by direct sequencing analysis. Of the 32 cell lines analyzed, the promoter hypomethylation of *MAGE-A1* and *MAGE-A3* was observed in both at 26 cell lines each. Further, 23 cell lines (SNU-61, SNU-175, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1197A, SNU-C1, SNU-C2A, Caco2, COLO201, COLO205, COLO320, DLD-1, HCT-15, HCT-116, HT-29, Lovo, LS174T, NCI-H716, SW403, SW1116, and WiDR) were simultaneously unmethylated in both the *MAGE-A1* and *MAGE-A3* genes. With exception, there were two cell lines (SNU-61, COLO201) with negative gene expression for either *MAGE-A1* or *MAGE-A3*, but unmethylated *MAGE-A1* or *MAGE-A3* promoter was detected. On the contrary, there were four cell lines (SNU-283, SNU-407, SNU-1047 and SW480) in which *MAGE* genes were strongly expressed, although no unmethylated *MAGE* promoter could be detected, suggesting the activation or inactivation of *MAGE* expression by another mechanism.

In our study, the rates of hypomethylation of the *MAGE-A1* and *MAGE-A3* genes in colorectal cancer cell lines were 81% in both and those in colorectal cancer tissues were 43% and 77%, respectively. The DNA was extracted from the surgically removed frozen-tissues;

however, the tumor tissues might have been contaminated with some normal stromal cells. Therefore, the levels of hypomethylation of *MAGE-A1* and *MAGE-A3* genes in cancer tissues might be affected by the DNA from normal cells. To obtain a better understanding of the promoter hypomethylation status of the *MAGE-A1* and *MAGE-A3* genes in colorectal cancer tissues, expression analysis of *MAGE-A1* and *MAGE-A3* (such as, *in situ* hybridization or immunostaining) and the more precise methylation analysis method (such as, laser capture microdissection techniques) to isolate cancer cells from normal cells need to be performed. The hypomethylation of the *MAGE-A1* and *MAGE-A3* genes in corresponding normal tissues was detected in only 2 and 5 samples (2% and 6%), respectively (Table 2).

To evaluate the association between the clinical parameters and *MAGE* expression, the Pearson χ^2 test was used to evaluate differences in tumor location (proximal or distal) or gender, and significance was determined using 95% confidence intervals. In our study, unmethylated *MAGE-A1* DNA expression was significantly different in respect of tumor location and gender. Unmethylated *MAGE-A1* DNA expression was significantly higher in distal location ($P = 0.028$) and in females ($P = 0.004$). However, unmethylated *MAGE-A3* DNA expression was not significantly associated with tumor location ($P = 0.239$), while it was only related to female gender ($P = 0.021$).

Our results supported the role of the promoter methylation in maintaining a silent phenotype of the *MAGE-A1* and *MAGE-A3* genes, as the *MAGE* gene was re-expressed after treatment with 5-aza-2'-deoxycytidine. This agent reactivates gene expression when methylation of CpG islands is the cause of reduced gene expression. We demonstrated that the *MAGE-A1* and *MAGE-A3* mRNAs were re-expressed after 5-aza-2'-deoxycytidine treatment in all 8 and 9 cell lines that did not express *MAGE-A1* and *MAGE-A3* mRNAs, respectively. However, the SNU-1040 and COLO 205 cell lines did not show re-expression, suggesting the inactivation of *MAGE-A1* expression by another mechanism.

In conclusion, we observed hypomethylation in the promoter region of both the *MAGE-A1* and *MAGE-A3* genes in 23 of 32 colorectal cancer cell lines. This methylation was confirmed by MS-PCR, treatment with 5-aza-2'-deoxycytidine, and bisulfite direct sequencing analysis. Hypomethylation of the promoter region appears to be a frequent phenomenon in human colorectal cancers and upregulates transcription of the *MAGE-A1* and *MAGE-A3* genes in colorectal cancer cells. In addition, out of 87 colorectal cancer tissues, we observed hypomethylation in the promoter regions of the *MAGE-A1* and *MAGE-A3* genes in 37 (43%) and 67 (77%) tissues, respectively. This suggests that promoter hypomethylation of *MAGE-A1* and *MAGE-A3* genes up-regulates its expression in colorectal carcinomas as well as in gastric cancers, and might play a significant role in the development and progression of human colorectal carcinomas.

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H pylori

***H pylori* infection and reflux oesophagitis: A case-control study**

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Abstract

AIM: To examine the relationship between *H pylori* and gastro-oesophageal reflux disease (GORD) in Iran.

METHODS: In this study 51 GORD patients (referred to endoscopy at Taleghani hospital) were compared with 49 age-sex matched controls. Diagnosis of *H pylori* was made by gastric mucosal biopsy and rapid urease test (positive if the result of one or both diagnostic methods was positive). Updated Sydney system was used to report histopathological changes.

RESULTS: The frequency of *H pylori* infection based on rapid urease test and histology was 88.2% (45) in patients and 77.6% (38) in controls, which showed no significant difference. The frequency of *H pylori* infection was significantly higher in the antrum than in the corpus and cardia. The mean activity, inflammation, and gastritis scores were also higher in the antrum of patients than in the antrum of controls. The mean scores were significantly higher in the corpus of controls than in the corpus of patients. Diffuse active gastritis was observed in a significantly larger number of controls, while the frequency of diffuse chronic gastritis was higher in patients. There was no significant difference in the frequency of other histological findings between patients and controls.

CONCLUSION: *H pylori* infection cannot prevent GORD in this region.

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Key words: *H pylori*; Gastro-oesophageal reflux diseases; Reflux oesophagitis

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INTRODUCTION

Heartburn is a common symptom in the general population^[1,2] and is associated with the development of adenocarcinoma of the oesophagus and cardia^[3]. Gastritis-associated hypochlorhydria may protect against gastro-oesophageal reflux disease (GORD)^[4,5]. It has hypothesized that the declined *H pylori* infection results in a decline in peptic ulcer and a concomitant increase in reflux disease and associated oesophageal adenocarcinoma^[6,7]. However, the relationship between *H pylori* infection and GORD has not been established^[8-11]. It was reported that prospective, large studies are needed to explore the *H pylori*-gastro-oesophageal disease relationship further and to avoid confusing potential benefits with known risks^[9-12].

The main of this study was to investigate whether there is a difference between the frequencies of *H pylori* infection in cases and controls, and the possible relationship between *H pylori* infection and GORD.

MATERIALS AND METHODS

Patients

In this study, patients with a history of heartburn, at least two times a week for a period of more than 3 mo, referred for gastrointestinal endoscopy at Taleghani Hospital, Shaheed Beheshti University of Medical Sciences, between March 2001 and February 2002, were enrolled. The reflux oesophagitis group included 51 patients (31 men and 20 women, mean age, 54.1 ± 17.2 years, range 17-80 years) with endoscopically diagnosed erosive reflux oesophagitis.

Table 1 Frequency of *H pylori* infection in two groups *n* (%)

Group	<i>H pylori</i>			Rut	Diagnosis of <i>H pylori</i>	Total
	Cardia	Corpus	Antrum			
Case	27 (52.9)	25 (49.0)	37 (72.5)	23 (45.1)	45 (88.2)	51 (100.0)
L.A. classification						
A	11 (21.6)	8 (15.7)	12 (23.5)	9 (17.7)	16 (31.4)	17 (33.3)
B	11 (21.6)	11 (21.6)	17 (33.4)	12 (23.5)	21 (41.2)	24 (47.1)
C	4 (7.8)	5 (9.8)	7 (13.7)	2 (3.9)	7 (13.7)	9 (17.7)
D	1 (1.9)	1 (1.9)	1 (1.9)	0	1 (1.9)	1 (1.9)
Control	28 (57.1)	27 (55.1)	31 (63.3)	22 (44.9)	38 (77.6)	49 (100.0)
Total	55 (55.0)	52 (52.0)	68 (68.0)	45 (45.0)	83 (83.0)	100 (100.0)

Patients with a history of upper gastrointestinal (GI) surgery, malignancy, oesophageal varices, and antibiotics or bismuth consumption during the last 6 mo, together with those using H₂ blockers, proton pump inhibitors (PPIs), alcohol, or non steroidal anti inflammatory drugs (NSAIDs) during the last 4 wk, were excluded from the study. The control group comprised: 49 asymptomatic patients (29 men and 20 women, mean age, 52.2 ± 17.1 years, range 18-80 years) without reflux oesophagitis, any symptom of upper GI diseases, and any lesion in their endoscopy, which was performed for other reasons (work up for iron deficiency, possible malignancy, and ERCP, sphincterotomy, or stone extraction candidates). Two cases of control group were missed during the study. The cases and controls were sex and age matched with a maximum difference of less than 3 years. Written informed consent was obtained for all upper endoscopy and biopsy procedures. This study was approved by the Ethics Committee of the Research Center of Gastroenterology and Liver Diseases, Shaheed Beheshti University of Medical Sciences.

Endoscopy and gastric biopsies

Endoscopy was performed for both case and control groups, by two endoscopists blinded to the status of the controls and patients. The presence and grading of reflux oesophagitis were determined according to L.A. classification, from A (least severe) to D (most severe)^[13]. During endoscopy, two biopsies were taken from the antrum, corpus, and cardia, and stained with standard haematoxylin/eosin and Geimsa to identify *H pylori* and histopathological changes. Rapid urease test was performed on the biopsy specimens from antrum, corpus, and cardia. The urease test was considered positive when the urea solution changed from yellow to pink at room temperature within 24 h. The diagnosis of *H pylori* infection was made by positive findings on either histology or urease test. Patients were considered to be *H pylori*-positive if the result of one or both diagnostic methods was positive and *H pylori*-negative if both methods revealed negative results.

For histopathological analysis, biopsy specimens were fixed in 40 g/L neutral-buffered formaldehyde and embedded in paraffin. Five-micron thick sections were cut from each paraffin block and stained with haematoxylin and eosin for routine histology. Two pathologists blinded to the clinical information of subjects assessed the histopathological changes independently. Updated Sydney

system was used to report histopathological changes^[14]. The degrees of inflammation and activity were scored from 0 (absent) to 3 (most severe). The inflammation score and activity score were summed and expressed as the gastritis score. The predominance for the anatomic regions in gastritis was determined based on the degree of inflammation in the different anatomic parts of the stomach. If the degree of inflammation was higher, the anatomic place with a higher grade of inflammation was stated as the predominant region. For the diagnosis of multifocal atrophic gastritis, we determined intestinal metaplasia or significant mucosal atrophy. Because of the low reproducibility for routine grading of mucosal atrophy, atrophy score was not used as a marker for grading^[14].

Statistical analysis

Results were expressed as mean ± SD. Odd's ratios (95% CI) were calculated to evaluate the differences in the frequency of *H pylori* infection and other histological findings between patient and control groups. Cochran's Q test was used to compare the frequency of *H pylori* infection in the cardia, corpus, and antrum. Mann-Whitney *U* test was used to analyze the differences in activity, inflammation, and gastritis scores between the two groups and Friedman test was used to analyze the different scores in the cardia, corpus, and antrum. *P* < 0.05 was considered statistically significant.

A sample size of about 50 for patient and control groups was considered to have an 80% detecting rate (at the two-sided 5% level) with at least a 25% difference in the prevalence of *H pylori* between the two groups.

RESULTS

Among the 51 reflux oesophagitis patients, 17 (33.3%) were in grade A, 24 (47.1%) in grade B, 9 (17.6%) in grade C, and 1 (1.9%) in grade D (Table 1). Hiatal hernia was observed in 30 (58.8%) patients. The prevalence of *H pylori* infection is shown in Table 1. The frequency of *H pylori* in the antrum was significantly higher than that in the corpus and cardia of the patients (*P* < 0.01), while the differences were not significant in different regions of stomach of the controls, which might be due to the inadequate sample size. We were not able to find any significant difference in the frequency of *H pylori* infection between the two groups (OR: 2.2, 95% CI: 0.7-7.4) as shown in Table 2.

The different histological findings in patients and

Table 2 Different histological findings in two groups *n* (%)

Histological finding	Case	Control	Total	OR (95% CI)
<i>H. pylori</i> infection ¹	45 (88.2)	38 (77.6)	83 (83.0)	2.2 (0.7-7.4)
Chronic inflammation in cardia	14 (27.5)	18 (36.7)	32 (32.0)	0.7 (0.3-1.7)
Chronic inflammation in corpus	8 (15.7)	22 (44.9)	30 (30.0)	0.2 (0.1-0.6)
Chronic inflammation in antrum	28 (54.9)	30 (61.2)	58 (58.0)	0.8 (0.3-1.8)
Overall gastritis categorization (Sydney classification)				
Diffuse chronic active gastritis	15 (29.4)	26 (53.1)	41 (42.0)	0.4 (0.2-0.9)
Cardia predominant chronic active gastritis	1 (2.0)	1 (2.0)	2 (2.0)	0.9 (0.0-36.3)
Corpus predominant chronic active gastritis	0	1 (2.0)	1 (1.0)	0.0 (0.0-16.9)
Antrum predominant chronic active gastritis	3 (5.9)	5 (10.2)	8 (8.0)	0.6 (0.1-2.9)
Multifocal metaplastic or atrophic gastritis	1 (2.0)	1 (2.0)	2 (2.0)	0.9 (0.0-36.3)
Chronic carditis	1 (2.0)	2 (4.1)	3 (3.0)	0.5 (0.0-6.9)
Diffuse chronic gastritis	10 (19.6)	2 (4.1)	12 (12.0)	5.7 (1.1-40.4)
Normal	20 (39.2)	11 (22.4)	31 (31.0)	2.2 (0.9-5.8)
Total	51 (100.0)	49 (100.0)	100 (100.0)	-

¹Based on histology and rapid urease test.

controls are shown in Table 2. The frequency of chronic inflammation in the corpus was significantly higher in controls than in patients (OR: 0.2, 95% CI: 0.1-0.6). Diffuse active gastritis was also observed in controls (OR: 0.4, 95% CI: 0.2-0.9), while diffuse chronic gastritis was observed in patients (OR: 5.7, 95% CI: 1.1-40.4). The frequency of intestinal metaplasia and mucosal atrophy was not significantly different between the two groups (Table 2).

The inflammation, activity, and gastritis scores in both groups are depicted in Table 3 and Figure 1. The mean activity score in the cardia, corpus, and antrum of controls was significantly higher than that of patients ($P < 0.01$ or $P < 0.001$, Figure 1A). The inflammation score was higher in the corpus of controls than that in patients ($P < 0.01$, Figure 1B), while the inflammation score of the cardia and antrum was not significantly different between the two groups. Similarly gastritis score was significantly higher in controls than in patients ($P < 0.01$ and $P < 0.05$, Figure 1C).

The mean activity score was significantly higher in the antrum than in the corpus and cardia of controls ($P < 0.001$, Table 3), while the differences were not significant in patients probably due to the inadequate sample size. The mean inflammation and gastritis scores were also significantly higher in antrum than in corpus and cardia of both patients and controls ($P < 0.001$, Table 3). These findings together with the higher frequency of *H. pylori* infection in the antrum indicated that *H. pylori* in antrum could induce inflammation.

DISCUSSION

Increasing attention has been paid to the relationship between *H. pylori* infection and reflux oesophagitis in recent years. GORD is a common condition affecting 25%-40% of the population^[2]. The presence of hiatal hernia^[15], transient relaxation of the lower oesophageal sphincter^[16,17], and impaired clearance of regurgitated gastric contents in the oesophagus^[18] are considered possible causative factors for GORD.

There is evidence that infection with *H. pylori* is the principal cause of peptic ulcer disease^[10]. However, there

Table 3 Scores of inflammation, activity, and gastritis in two groups

Group	Cardia	Corpus	Antrum	<i>P</i>
	Median (range)			
Case				
<i>H. pylori</i> <i>n</i> (%)	27 (52.9)	25 (49.0)	37 (72.5)	0.005 ¹
Activity score	0 (0-2)	0 (0-2)	0 (0-3)	0.057 ²
Inflammation score	1 (0-2)	1 (0-2)	2 (0-3)	0.000 ²
Gastritis score	0 (0-4)	1 (0-4)	2 (0-6)	0.000 ²
Control				
<i>H. pylori</i> <i>n</i> (%)	28 (57.1)	27 (55.1)	31 (63.3)	0.465 ¹
Activity score	1 (0-2)	1 (0-3)	1 (0-3)	0.000 ²
Inflammation score	1 (0-3)	1 (0-3)	2 (0-3)	0.000 ²
Gastritis score	2 (0-5)	2 (0-6)	3 (0-6)	0.000 ²

¹Cochran's Q test; ²Friedman test.

is uncertainty about the role of this organism in GORD and the available data do not demonstrate an evident association between these two factors, although an etiologic link has been found between *H. pylori* infection and GORD or peptic oesophagitis^[10]. The prevalence of *H. pylori* infection in patients with GORD in our study (88.2%) was higher than that reported in other studies^[9], suggesting that *H. pylori* infection is more frequent in developing countries than in industrialized countries^[19].

No difference was found in the prevalence of *H. pylori* between patients with reflux oesophagitis and controls in this study. Conflicting evidence about the association of *H. pylori* infection with GORD has been reported and geographical location is an important determinant^[9]. The pathogenic role of *H. pylori* in reflux oesophagitis is suspected in earlier studies^[20] while other studies have found no relationship between *H. pylori* prevalence in GORD patients with that reported in other patients^[10,21-34]. In contrast, the possible protective role of *H. pylori* in reflux oesophagitis and other GORD-related diseases such as Barrett's oesophagus and oesophageal adenocarcinoma has recently been suggested^[35-39]. *H. pylori* can cause chronic gastritis in virtually all infected people. This persistent inflammation ultimately leads to loss of the normal architecture of gastric mucosa, disappearance of gastric

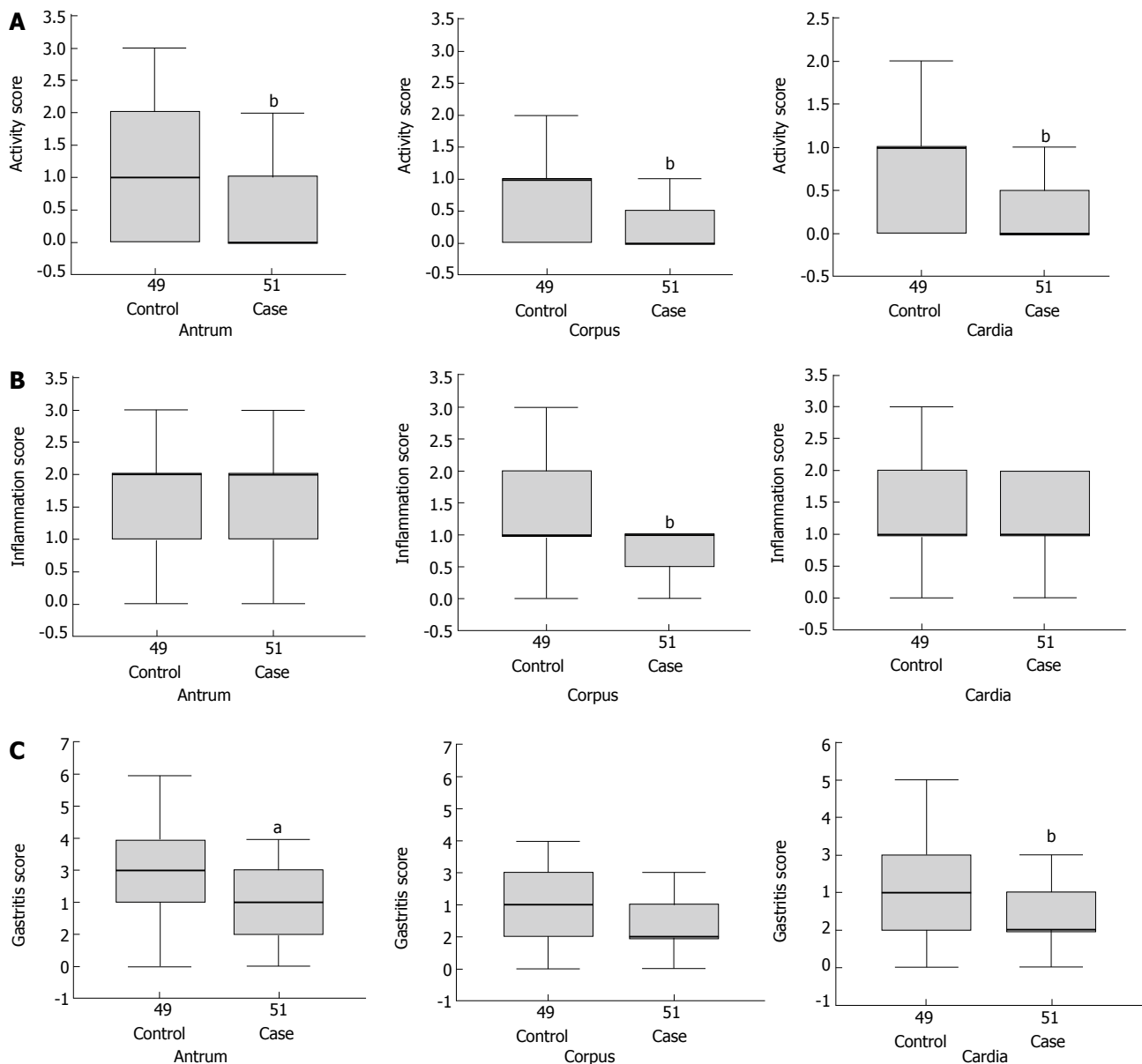


Figure 1 Scores of activity (A), inflammation (B), and gastritis (C) in the antrum and corpus of patients and controls. ^a $P < 0.05$, ^b $P < 0.01$ vs controls.

glands and specialized cells^[40]. Diffuse active gastritis was observed in controls while diffuse chronic gastritis was observed in patients in the present study, suggesting that active inflammation might play a protective role in GORD. Chronic antrum-predominant gastritis has been shown to be associated with secretion of acid and formation of duodenal ulcer^[8,41]. In patients otherwise predisposed to reflux disease, antrum-predominant gastritis may therefore increase acid production and reflux disease development^[8]. On the other hand, atrophy induced by chronic *H. pylori* infection (chronic corpus gastritis) with decreased gastric acid production can protect against reflux oesophagitis^[8,11,42]. As a consequence, the antrum predominant inflammation might be considered a factor for *H. pylori* infection.

In conclusion, *H. pylori* infection is not associated with DORD. Multicentre prospective studies with a larger sample size are needed to explore the relationship between *H. pylori* infection and DORD.

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Natural maternal transmission of *H pylori* in Mongolian gerbils

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Abstract

AIM: To investigate maternal *H pylori* infection status to determine the potential of maternal transmission.

METHODS: In the present study, we examined these issues in an experimental murine model, which is a Mongolian gerbil model that has been reported as an optimal laboratory animal model to study *H pylori*. Pregnant Mongolian gerbils, infected experimentally with *H pylori*, were divided into as four groups. Following the experimental design, the stomachs of the mother and litters were isolated and assessed for transmission of *H pylori* at the prenatal period, parturition day, 1-wk old and 3-wk old respectively. Bacterial culture and polymerase chain reaction (PCR) were used to examine the presence of transmitted *H pylori*.

RESULTS: All litters showed no transmission of *H pylori* during pregnancy and at parturition day. However, they revealed 33.3% and 69.6% at 1-wk and 3-wk of age respectively by PCR.

CONCLUSION: These results suggested that vertical infection during the prenatal period or delivery procedure is unlikely as a route of mother-to-child *H pylori* infection. It may be that *H pylori* is acquired through breast-feeding, contaminated saliva and fecal-oral transmission during co-habitation.

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Key words: *H pylori*; Vertical; Maternal; Transmission

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INTRODUCTION

H pylori is a gram-negative, spiral-shaped, microaerophilic bacterium that infects the human gastric mucosa^[1]. Chronic infection is thought to be associated with chronic active gastritis, peptic ulcer and gastric malignancies, such as mucosa-associated B cell lymphoma and adenocarcinoma^[2-4]. In particular, this organism has been categorized as a class I carcinogen by the World Health Organization^[5] and previous studies have confirmed that long-term infection with *H pylori* induces adenocarcinoma in Mongolian gerbils^[6,7]. In-depth knowledge of the transmission patterns may constitute important information for future intervention strategies. In the absence of consistent and verified environmental reservoirs, a predominantly person-to-person transmission has been postulated. *H pylori* infection is associated with poor living conditions, and possible transmission routes are fecal-oral, oral-oral, or gastro-oral, but firm evidence is lacking^[8-11]. Young children are particularly vulnerable to infection by transmission of *H pylori* from their infected parents, especially infected mothers^[12-15], and it is generally believed that such transmission is influenced by socio-economic status^[16,17]. However, little is known about how and when maternal transmission occurs during the perinatal period, especially whether this occurs before or after parturition. In the present study, we examined these issues in an experimental murine model, which is a Mongolian gerbil model that has been reported as an optimal laboratory animal model to study *H pylori* *in vivo*^[18].

The present study was designed to examine the incidence of vertical transmission of *H pylori* from their infected mother during the perinatal period in an experimental murine model.

MATERIALS AND METHODS

Experimental design

The experimental scheme of this study was summarized in Figure 1. Pregnant Mongolian gerbils were infected experimentally with *H pylori*. The stomachs of the litters were isolated and assessed for transmission of *H pylori* during pregnancy and at parturition day, 1 wk and 3 wk after delivery respectively. Their mother was also evaluated for the infectious status of *H pylori*. To determine the vertical transmission of *H pylori*, bacterial culture assay and polymerase chain reaction (PCR) were conducted with each sample.

Animals

Specific pathogen-free (SPF) 3-mo-old male and female

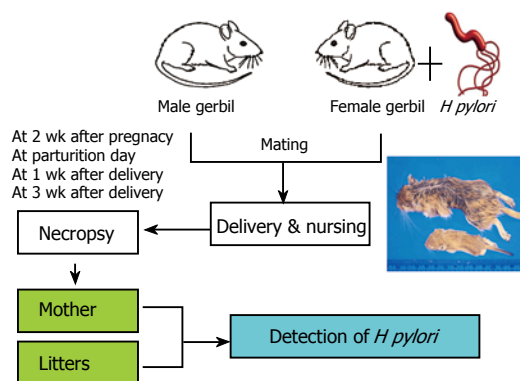


Figure 1 Scheme of experimental vertical transmission.

Mongolian gerbils (*Meriones unguiculatus*) were obtained from the SPF Animal Facilities of College of Medicine, Seoul National University, South Korea. All animals were kept in the inspecting facility of Wonkwang University (Iksan, South Korea) for 1 wk before experimentation to allow acclimation. Thereafter, they were kept in an isolated SPF barrier room with regulated temperature ($23^{\circ}\text{C} \pm 1^{\circ}\text{C}$), humidity ($50\% \pm 5\%$) and light/dark cycle (12/12 h). The animals were fed a sterilized pellet diet by 2 M rad radiation (Purina, Korea) and sterilized water *ad libitum*. All studies were performed in accordance with the Guide for Animal Experimentation by Wonkwang University and approved by the Institutional Animal Care and Use Committee of Wonkwang University (Iksan, South Korea). All efforts were made to minimize pain or discomfort of animals used.

Preparation of *H. pylori* & inoculation

H. pylori (ATCC 43504; American Type Culture Collection, USA) was incubated in a brain-heart infusion broth containing 10% fetal bovine serum at 37°C overnight under a microaerophilic atmosphere and allowed to grow to a density of 2.0×10^9 colony-forming units (CFU) per 1 mL of culture broth. Animals were inoculated twice at 3-d intervals by oral administration of 1.0×10^9 CFU of *H. pylori* suspended in 0.5 mL of broth. The challenged animals were confirmed to be *H. pylori*-positive by PCR of their fecal samples as described previously^[19]. *H. pylori*-negative animals were excluded from the following study.

Maternal transmission of *H. pylori*

One week after the challenge with *H. pylori*, the infected females and males were transferred to separate cages for mating. As soon as a female was confirmed to be pregnant, she was separated from the group and cared for until delivery. Twelve infected pregnant females were used to determine maternal transmission. Three mothers were sacrificed at 2 wk after pregnancy. The gastric samples of mother and fetuses were isolated and submitted to determine *H. pylori* infection. Also, another nine pregnant gerbils were cared for until delivery and the mother and her litters were housed in one cage per family. For 12 h before their sacrifice, they were housed in a grated cage and deprived diet (Figure 2). The stomachs of the mother and litters were isolated and assessed for transmission of



Figure 2 The mother and her litters were housed in one cage per family. For 12 h before their sacrifice, they were housed in grated cage and deprived diet.

H. pylori at parturition day, 1 wk and 3 wk after delivery respectively. For the negative control, an uninfected female and her litters were sacrificed at 2 wk after pregnancy, parturition day, 1 wk and 3 wk after delivery respectively. Thereafter their gastric samples were submitted to examine *H. pylori* infection.

Isolation of *H. pylori*

Aliquots of homogenate were cultured on M-BHM *pylori* agar medium plates and the plates were incubated under the previous described condition^[19]. To confirm *H. pylori* infection, the remainder of the homogenate was used for the following PCR procedure.

Polymerase Chain Reaction

Bacterial DNAs were extracted from the above homogenate by bead beater-phenol extraction method^[19]. Each sample homogenate was suspended in 200 μL of Tris-EDTA-NaCl buffer [10 mmol/L Tris-HCl, 1 mmol/L EDTA, and 100 mmol/L NaCl (pH 8.0)]. A bacterial suspension was placed in a 2.0-mL screw-cap microcentrifuge tube filled with 100 μL (packed volume) of glass beads (diameter, 0.1 mm; Biospec Products, USA) and 100 μL of phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma Chemical Co., USA). The tube was oscillated on a Mini-Bead Beater (Biospec Products) for 1 min and was centrifuged ($12000 \times g$, 5 min) to separate the phases. The aqueous phase was subsequently transferred into another clean tube; 10 μL of 3 mol/L sodium acetate and 250 μL of ice-cold absolute ethanol were added. To precipitate the DNA, the mixture was kept at -20°C for 10 min. The harvested DNA pellets were dissolved in 60 μL of Tris-EDTA buffer [10 mmol/L Tris-HCl and 1 mmol/L EDTA (pH 8.0)] and were used as a template DNA for PCR. A set of primers (HF, 5'-ACTTTAAACGCATGAA GATAT-3'; and HR, 5'-ATATTTTGACCTTCTGGGGT -3') was used to detect specific nucleic acid of *H. pylori*^[19]. The template DNA (50 ng) and 20 pmol of each primer were added to a PCR mixture tube (Maxime PCR PreMix; iNtRON Biotechnology, Korea) containing 1 U of Taq DNA polymerase, 250 $\mu\text{mol/L}$ each deoxynucleoside triphosphate, 50 mmol/L Tris-HCl (pH 8.3), 40 mmol/L KCl, 1.5 mmol/L MgCl_2 , and the gel loading dye. The volume was adjusted with distilled water to 20 μL . The

Table 1 Results of cultures and PCR for assessment of transmission of *H pylori* during pregnancy and at parturition day

Infection status	Evaluated time	Subject	Detection rate of <i>H pylori</i> (No. of positive/No. of animal)	
			Culture	PCR
Infected female	Pregnancy	Mothers	3/3	3/3
		Fetuses	0/23	0/23
	Delivery	Mothers	3/3	3/3
		Litters	0/21	0/21
	1-wk old	Mothers	3/3	3/3
		Fetuses	5/21	7/21
	3-wk old	Mothers	3/3	3/3
		Litters	11/23	16/23
Uninfected female	Pregnancy	Mothers	0/1	0/1
		Fetuses	0/7	0/7
	Delivery	Mothers	0/1	0/1
		Litters	0/9	0/9
	1-wk old	Mothers	0/1	0/1
		Litters	0/7	0/7
	3-wk old	Mothers	0/1	0/1
		Litters	0/7	0/7

reaction mixture was subjected to 30 amplification cycles (5 min at 95°C, 30 s at 94°C, 30 s at 52°C, 45 s at 72°C, and 5 min at 75°C) followed by a 5-min extension at 72°C (model 9600 Thermocycler; Perkin-Elmer Cetus, USA). The PCR products were electrophoresed on a 1.2% agarose gel.

RESULTS

While culture of the bacterium is one of the gold standards in the diagnosis of *H pylori* infection, in the present study, we also used the PCR with the culture method to detect *H pylori*. The culture method was not considered to be ideal for determination of *H pylori* transmission because only small amounts of bacteria were suspected to colonize the stomach and the detection limit of the quantitative culture assay was 1×10^2 CFU/g gastric tissue^[20]. The sensitivity of the PCR was 1 CFU/g feces and higher than those of other assays^[19]. Vertical transmission was examined at 2 wk after pregnancy and at parturition day (corresponding to the transplacental or intrauterine transmission during prenatal period and the delivery transmission during birth canal passage, respectively). Each stage group was composed of three pregnant females and their litters.

As the results of assessment performed at 2 wk after pregnancy, the mothers revealed *H pylori* infected status by culture assay and PCR (Table 1). However, their fetuses were not infected with *H pylori* (Figure 3). For the evaluation of delivery transmission during birth canal passage, all litters showed no transmission of *H pylori* (Figure 4) although the mothers were identified *H pylori* infected by culture assay and PCR (Table 1). However, their fetuses were not infected with *H pylori* (Table 1). *H pylori* was not detected in any litters and their mothers of the negative control group (Table 1).

Maternal transmission was examined at 1 wk and 3 wk postpartum (corresponding to the transitional milk and



Figure 3 Amplification of *Helicobacter* DNAs. All fetuses showed no transmission of *H pylori* at 2 wk after pregnancy. P: Positive control; N: Negative control; Mo: Mother; F: Fetuses.

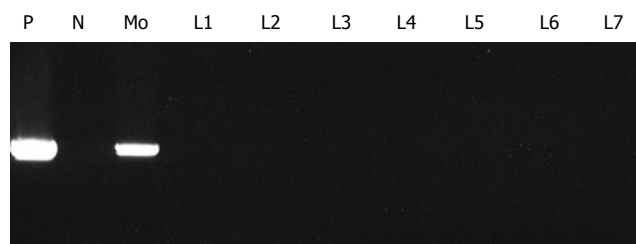


Figure 4 Amplification of specific nucleic acids for *H pylori*. All litters showed no transmission of *H pylori* at parturition day. P: Positive control; N: Negative control; Mo: Mother; L: Litters.

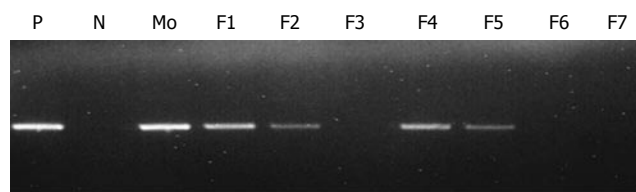


Figure 5 At 3 wk postpartum, some of litters delivered from infected mothers revealed positive reaction. P: Positive control; N: Negative control; Mo: Mother; L: Litters.

weaning stage, respectively). Each stage group was composed of three families. At 1 wk and 3 wk postpartum, the mothers revealed *H pylori* infected status by culture assay and PCR and some of their litters were infected with *H pylori* (Figure 5). The frequency of maternal transmission was increased during the nursing period. The transmission rate at 3 wk postpartum was significantly higher than at 1 wk postpartum (Table 1). *H pylori* was not detected in any litters of the negative control group.

DISCUSSION

Half of the world's population is estimated to be infected with *H pylori* and the infection is mainly acquired in early childhood but the exact routes of transmission remain elusive. Infected mothers are generally considered to be the main source of the pathogen^[15,21,22]. The epidemiology of *H pylori* infection is variable, with prevalence being significantly higher and incident infection occurring earlier in developing countries compared with developed countries^[17,23,24]. There is an obvious public health impact of *H pylori* infection and thus, to design targeted and cost-effective prevention strategies, elucidation of the mode of transmission for this bacterium is crucial^[25]. It

is known that *H pylori* infection is typically acquired in early childhood and usually persists throughout life unless specific treatment is applied^[12,14,26]. Definitive modes of transmission have not yet been characterized and the principal reservoir appears to be humans. Person-to-person transmission via fecal-oral, oral-oral and gastro-oral routes have been proposed^[8-11]. Numerous studies also indicate low socioeconomic status, including domestic overcrowding in childhood, as major risk factors for higher infection prevalence rates^[16,17,23,27,28]. Little is known about when and how often maternal transmission of *H pylori* occurs during the perinatal stage. In the present study, we examined these issues in an experimental murine model.

The results of the vertical-transmission experiment indicated that vertical transmission of *H pylori* did not occur at the pregnant and delivery stage. However, they revealed 33.3% and 69.6% at the lactating and weaning stage respectively. Recent epidemiological studies in humans suggest that acquisition of *H pylori* occur during childhood. For example, Rothenbacher *et al*^[29] reported that *H pylori* acquisition seems to occur mainly between the first and second year of life: that is, after the age of weaning. Our results are in agreement with this report. Also, Rothenbacher *et al*^[22] reported that infected parents, especially infected mothers, play a key role in the transmission of *H pylori* within families. Maternal contact behaviour during the breastfeeding period may be responsible for the high frequency of maternal transmission^[30]. Our results also showed that the maternal-transmission of *H pylori* was not observed during pregnancy and delivery stage, but detected at lactating and weaning stage. On the basis of these findings, vertical infection during pregnancy or at delivery is unlikely as a route of mother-to-child *H pylori* infection. We suggested that *H pylori* infection by the transplacental route during pregnancy does not occur and that *H pylori* transmission by discharges of the uterine or vagina, obstetric delivery tract, during parturition does not occur. *H pylori* might be acquired through breast-feeding, contaminated saliva and fecal-oral transmission during co-habitation.

In conclusion, the present study provides new and important information on maternal transmission of *H pylori*. This study implied that maternal transmission of *H pylori* might be developed during latency or a later postpartum stage. Data from human children are limited, because most *H pylori*-infected children have no symptoms and it is difficult for a paediatrician to examine such asymptomatic children invasively. In the present study, we examined these issues in an experimental murine model, the Mongolian gerbil model that has been reported as an optimal laboratory animal model to study *H pylori in vivo*^[18]. We analysed the stomachs of many infant Mongolian gerbils directly and we believe that it is meaningful to use our results to speculate when *H pylori* infection occurs in human children. The acquisition of *H pylori* infection during childhood seems to be a critical risk factor for the later development of gastric cancer. The prevention of transmission of *H pylori* during childhood could provide an effective strategy to decrease *H pylori* infection and gastric cancer.

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BASIC RESEARCH

Mechanical behavior of colonic anastomosis in experimental settings as a measure of wound repair and tissue integrity

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ues to anastomotic failure.

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Abstract

AIM: To determine the mechanical properties of anastomotic colonic tissue in experimental settings and therefore give a measure of wound healing.

METHODS: Thirty-six male Wistar rats were used as experimental models of anastomotic tissue integrity. On the 5th post-operative day, the tensile strength was measured by application of an axial force, providing a quantitative measure of anastomotic dehiscence and leakage.

RESULTS: Diagrams of the load as a function of the time [$P = P(t)$] and of the displacement also as a function of time [$\Delta s = \Delta s(t)$] were recorded for each test, permitting the design of the load versus the displacement diagram and thus providing significant data about the critical values of anastomotic failure. Quantitative data were obtained concerning the anastomotic strength of both control specimens (healthy rats), as well as specimens from non-healthy rats for comparison.

CONCLUSION: This experimental model provides an excellent method of measuring anastomotic strength. Despite the relative small number of specimens used, this method provides an accurate way of measuring wound repair. More experimental measurements need to be performed to correlate emerging tensile strength val-

INTRODUCTION

Investigating wound healing and attempting to improve its outcome necessitates process quantification^[1]. Parameters for anastomotic repair and adhesion formation^[2] may be mechanical, biochemical, or histological. Histology is not a primary tool for quantification when comparing various series of experimental anastomoses. Certainly it is very useful to describe the course and eventual result of the healing sequence at the tissue level. Also, the successive infiltration of various cells into the wound area may be followed, and obvious differences between anastomoses (e.g., ileal and colonic) will certainly be demonstrated this way. However, the measurement of choice to evaluate anastomotic repair and the effects of variations in surgical techniques, administration of drugs, or of any other modification to establish procedures, will mostly be either mechanical or biochemical or both.

The developing mechanical strength is, without doubt, a meaningful parameter to follow while investigating anastomotic healing. For this purpose, two fundamentally different approaches can be chosen. First, one can choose bursting strength, which is expressed either as bursting pressure or bursting wall tension, which is the measure of the resistance of the intestinal wall to increasing intraluminal pressure. Second, one can choose breaking strength, which reflects the resistance of the intestinal wall to forces exerted in a longitudinal direction^[3-5].

While both of these methods used to evaluate anastomotic healing have been investigated in the international literature, no paper has so far described

in detail the process itself, analyzing its advantages, disadvantages and parameters taken into consideration, therefore establishing the need for an in-depth presentation of the mechanical apparatus used and presenting not only the theoretical background behind the measurements but also the technical difficulties that arise.

MATERIALS AND METHODS

Thirty-six male Wistar rats weighing 300-350 g were used, and were housed two per cage. They were fed a standard diet and water *ad libitum*. All experiments were approved by the Athens Prefecture, Directorate of Veterinary Services (License No. K/355/27-1-2005), according to the Presidential Decree No. 160/1991 (Governmental Gazette A' 64), with which Greece has conformed to the 86/609/EEC directive. Laparotomy^[6] was performed through a midline 2 cm incision under anesthesia induced by ketamine (80 mg/kg) and xylazine (3 mg/kg). A colonic segment, 1 cm in length, 5 cm distal to the ileocecal junction was transected and the colon was re-anastomosed end-to-end using 5-0 Vicryl (Ethicon) sutures in single-layer interrupted fashion^[7]. About 10 sutures were placed for each anastomosis to secure an inverted anastomosis without mucosal protrusion, which is regarded as a major cause of perianastomotic adhesions. The abdominal muscle wall was then closed with 5-0 Vicryl (Ethicon) sutures, followed by skin closure with 4-0 Silk (Medipac) sutures.

To obtain the test specimen, the rats were sacrificed with an overdose of ether, on the 5th post-operative day. The previous abdominal incision was reopened, and the anastomotic site identified and inspected for possible adhesions and leakage. An 8 cm segment of the colon with the anastomosis in the middle was resected. Care was taken not to detach adhesions from the anastomosis, but to dissect the surrounding tissues. The resected specimen was gently irrigated with saline to remove feces and was mounted on a table.

The basic purpose of the present experimental protocol is the determination of the mechanical behavior of intestinal anastomoses and more specifically the response to tensile loading and the determination of the respective tensile strength. One can define the ratio of the applied force at the moment of failure, F_{cr} , over the surface, A , upon which the force acts normally^[8], as tensile strength (Figure 1). The ratio of the force over the respective area is known in engineering science as stress and therefore the tensile strength is the respective tensile stress at the moment of failure.

In the international scientific literature the mechanical behavior under tension, of specimens like the ones of the present protocol, has been studied in two ways: (1) By applying an internal hydraulic pressure, p ,^[9-12]. In this case (and for points relatively far from the borders of the specimens) a stress state equivalent to the so-called biaxial tension appears on the surface of the concave cylindrical specimen. Assuming that the thickness of the specimens is much smaller in comparison to its diameter, the principal tensile stresses at the moment of failure are given by the

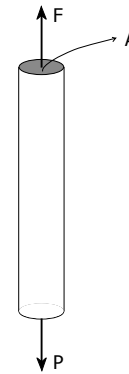


Figure 1 Typical forces applied on a standard specimen.

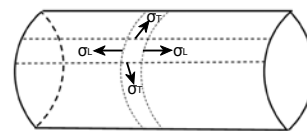


Figure 2 The application of hydraulic pressure creating a two-dimensional stress field.

relations (Figure 2)^[13]:

$$\sigma_L = \frac{p_{cr}r}{2t}, \quad \sigma_T = \frac{p_{cr}r}{t}$$

where p_{cr} is the value of the hydraulic pressure at the moment of the first failure, r the radius of the intestine and t its wall thickness. The stress system is characterized as principal since no shear stresses can be generated by a pure hydraulic pressure.

(2) By applying directly an axial force, F ,^[14,15]. In this case the critical value of the tensile strength is expressed as:

$$\sigma_{cr} = \frac{F_{cr}}{2\pi r t}$$

The procedures described above present both advantages and disadvantages. More specifically, the application of hydraulic pressure, although relatively easily realizable in the laboratory, creates a two-dimensional stress field, as shown in Figure 2. Therefore the conclusions drawn are not directly comparable to the respective ones of the experiments with uniaxial loading. Especially in the case of anisotropic materials (like the ones of the present study), it is impossible to define which one of the two stresses is responsible for the failure and therefore it is not possible to determine the critical value, since the direction at which failure will appear is not *a priori* known.

On the contrary, the application of an axial force is especially difficult from a practical point of view (as it will be seen in the next paragraph), but the results obtained are directly usable without reductions and additional assumptions.

Experimental difficulties of the direct tension experiment

In the present study the second procedure (direct tension) was adopted. The most important difficulties stated above

are summarized as following: (1) The nature of the materials under study renders the gripping of the specimens, with the aid of conventional friction grips through compression loads, extremely difficult. In fact, since it is impossible to form “gripping heads” to the specimens (“dog-bone” specimens), it is given that the failure will appear in the portion of the specimen which is inside the grips or in their immediate vicinity. However, in this area the stress field is strongly triaxial and therefore the results obtained are invalid and should be rejected. On the other hand, the limited chances to obtain long specimens in combination with the low friction coefficient between the external surfaces of the specimens (intestines) do not allow the use of pulley-shaped grips, in which the holding force emanates from the friction of a number of successive layers of the material rolled around the periphery of the pulley. (2) The extremely low force which is necessary for the fracture of even intact and healthy specimens, which according to international literature is estimated at the value of a few tenths of Newton^[14], renders the conventional arrangements of applying axial tension practically useless. (3) The nature of the specimens under study, which are twisted and bended around different axes, due to adhesion formation around the anastomotic area, renders the measurements of length changes and therefore of reduced deformations (strains, ϵ) almost impossible. (4) Finally, the nature of the intestines once again, which under torsion are “self-configured” into the form of plane plates, results in an interaction between the walls of the specimens, making difficult the reduction of the external loads into stresses (σ). Another factor making the situation more difficult is the non-constant thickness of the specimens throughout their length and their perimeter, which does not allow us to calculate the effective area of the loaded intestine.

In order to confront these difficulties in the present experimental study, the following procedures were adopted.

Gripping the specimens

A specific gripping system was designed, consisting of a pair of light metallic pins of cylindrical cross-section of diameter equal to 5 mm, with rounded head which permits easy entrance of the intestine in the pin, without injuring the specimen walls, reducing thus the time required for the in-situ preparation of the specimens (Figure 3). The pins are grooved at their mid-length and a suture which holds the specimens in place is rolled up in this groove. The upper part of the pins is drilled through the thickness and the specimen is suspended through this hole from the upper plate of the loading frame. At the same time, the second pin is fixed to the immobile plate of the frame.

The suspension and fixing of the pins is achieved with the aid of circular rings. In this way the maximum possible number of degrees of freedom is given to the specimen making possible the self-alignment and the “untwisting” of the intestine during tension without external limitations and therefore without, as much as possible, the development of parasitic tensions and disfigurations.

Despite the low total weight of the specimens gripping system (about 0.12 N), it was deemed appropriate to add the weight of the lower half, which is suspended

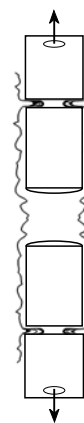


Figure 3 The gripping system consisting of a pair of light metallic pins. The grooves at which the intestine is gripped are indicated by the arrows.

and therefore sustained by the specimen, at the value of the final failure load, taken into consideration that these values are relatively comparable (the mean value of the failure load, as it was obtained from a series of preliminary experiments is equal to about ranges between 1.00 N and 2.20 N).

The load application system

After the rejection of loading through the application of dead weights (water or lead grains), due to the induction of vibrations and oscillations, a special load cell of capacity of 5 N and sensitivity of 10^{-3} N was used attached in a stiff electrical loading frame (Instron). This frame was selected, apart from its robustness, because it provides the ability of choosing the load application speed between wide ranges (from 0.5 mm/min to 500 mm/min). This characteristic of the frame is very important in case biological materials are to be studied, since their mechanical behavior exhibits viscoelastic nature, which is strongly dependent on the strain rate induced ($d\epsilon/dt$).

In the first phase of the experimental project an especially low tension speed (1 mm/min) was selected, and therefore the loading can be considered as static or at least quasi-static. As a result the overall duration of each test usually exceeds 10 min. It is planned, in a second phase, to study the effect of loading rate by employing dynamic or quasi-dynamic protocols.

Calibration of the apparatus

Before starting the main series of experiments, a number of preliminary tests were carried out, in order to define the range of the expected values of both the failure load and the elongation of typical specimens, and to calibrate the apparatus in the specific range of values.

The calibration of the loads was achieved with the safest method of the suspension of standardized (certified) weights from the load cell. Both the absolute reading values of the load cell as well as their linearity at the range of the expected loads were checked. The deviations detected for the absolute values of the loads did not exceed in any case the limit of 0.2% set by the “Quality Assurance System” of the Laboratory of Testing and

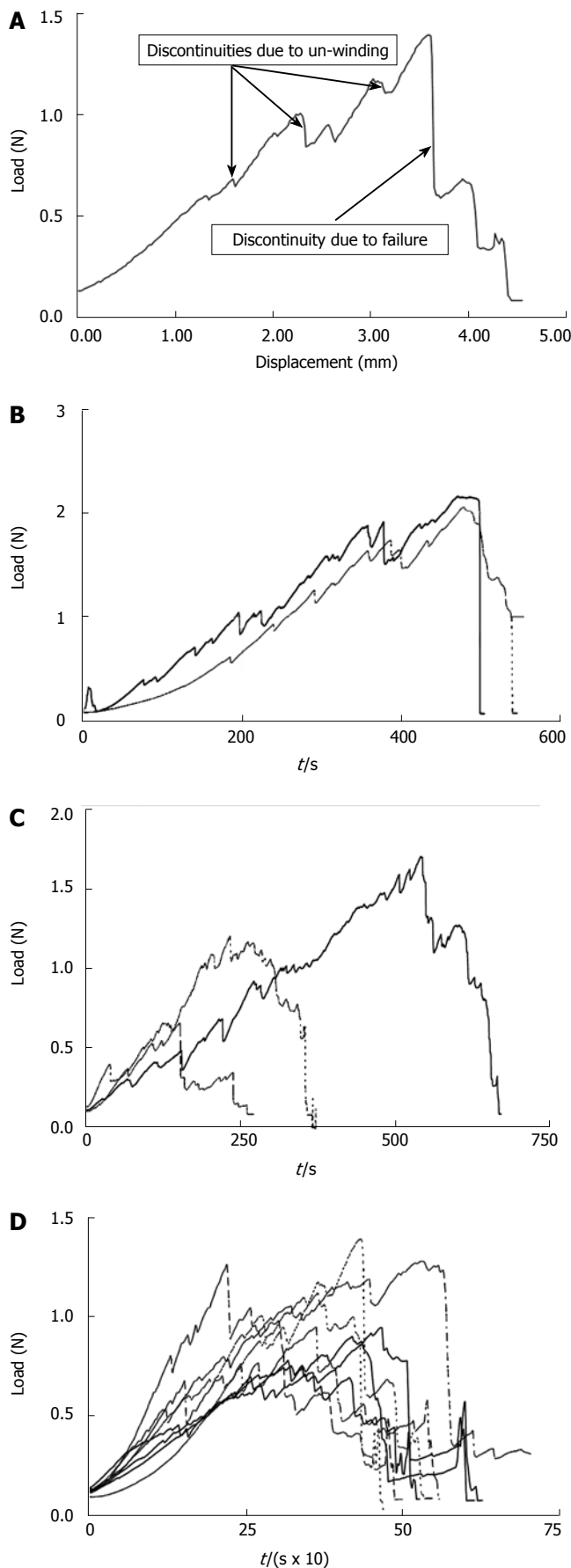


Figure 4 Load versus time and displacement diagrams for characteristic tests. **A:** Load versus displacement for a typical test of the preliminary series; **B:** Load versus time using intact specimens from healthy rats; **C:** Load versus time using specimens from healthy rats after colonic anastomosis; **D:** Load versus time using specimens from non-healthy rats after colonic anastomosis.

Materials of the National Technical University of Athens (NTUA/LTM), as it is described in the respective "Quality Assurance Manual" according to ISO9000/2000 system.

On the other hand, the linearity of the values of the loading cell in relation to the respective ones of the standard weights exceeded 99.8% for the whole range of interest, as it was concluded from a linear interpolation in the experimental data, using the least square method.

The calibration of the readings of the load frame for the displacements was achieved with the aid of three LVDT's (Linear Voltage Displacement Transducers), which have been verified with a standard micrometric vernier of an accuracy of 1 μm . Apart from the absolute values of the displacements, the parallel of the motion of the loading frame was also checked. The deviations detected did not exceed in any case the limits set by "Quality Assurance System" of the NTUA/LTM. Finally, the time recording device of the data acquisition and storage system was also calibrated with the aid of a prototype chronometer. The deviations were not measurable.

Data acquisition and storage system

The data to be recorded during the experiments include the values of the load as a function of the time [$P = P(t)$] and the values of the displacement of the moving plate of the loading frame also as a function of time [$\Delta s = \Delta s(t)$]. The data acquisition system includes a special multi channel "bridge" (National Instruments, type SCXI-1000), with the ability of adjusting the sampling rate. The system includes, also, a personal computer with suitable commercial software (LabVIEW-8). From the functions $F = F(t)$ and $\Delta s = \Delta s(t)$ recorded, one can eliminate the time obtaining the function of the applied force as a function of the displacement induced and therefore as a function of the elongation of the intestine, i.e. $F = F(\Delta s)$.

After the preliminary experiments, it was deemed appropriate to add to the data acquisition system a video device, in order to monitor the specimen during the experiment in a mode synchronous to the recording of the values of the load and the displacement. This was considered necessary, since the records of the load versus presented oscillations, due to two different reasons: (1) The "un-twisting" of the twisted parts or the "un-folding" of the folded parts of the intestine, which lead to a sudden length increase of the specimen, and therefore to instantaneous unloading, that is to a fall in the recorded load, as it is shown characteristically in the diagram of Figure 4A. (2) Local failures of parts of the specimen and especially in the case of anastomosed intestines failure of the anastomotic area itself or of directly neighboring areas, due to the tearing of the material from the anastomotic suture.

Since one cannot distinguish between these two discontinuities of the $F = F(t)$ diagram, the synchronous video-recording of the experiment was considered necessary. In this way it is possible to locate the discontinuities of the diagram due to the "un-twisting" or to the "un-folding" of the specimen, until the discontinuity due to the anastomotic failure or failure of its immediate neighboring area. Therefore the loading corresponding to this discontinuity can be safely considered as the crucial anastomotic failure load.

RESULTS

Three different classes of specimens were tested using the system described in the previous paragraphs.

The first one included a number of “intact” specimens, i.e. specimens from healthy rats without anastomoses. The results of these tests are to be used as a measure that will permit the characterization of the quality of the anastomosis, at least from the point of view of mechanical strength. Two characteristic examples of these tests are shown in Figure 4B. The data obtained from these tests for the failure force exhibited very small scattering (as it was perhaps expected) and the average value was of the order of:

$$F_{cr}^{intact} = 2.09 \text{ N} \pm 0.6 \text{ N}$$

Taking into account that the thickness of the wall of the intestine of the rats after the 8th week of their life is stabilized to about 1.1 mm while its perimeter varies in the range 9-12 mm^[16], it is concluded that the tensile failure strength of the “intact” specimens ranges between:

$$160 \text{ kPa} \leq \sigma_{fail}^{intact} \leq 210 \text{ kPa}$$

The second class of experiments included the control tests, namely it was carried out using specimens obtained from healthy rats but after having been subjected to colon anastomoses. The scattering of this series of tests was obviously higher compared to that of the “intact” specimens and it was considered necessary to study the acceptability of the results based on statistical experiments. The Chauvenet criterion was adopted and a number of tests were excluded from the analysis. In Figure 4C the results of three tests of this series are shown, corresponding to the ones with the lowest and highest acceptable failure forces and to a third one with failure force almost equal to the average value. The average value for the failure force was determined equal to:

$$F_{fail}^{control} = 1.35 \text{ N} \pm 0.42 \text{ N}$$

Similarly the failure strength ranges between:

$$100 \text{ kPa} \leq \sigma_{fail}^{control} \leq 135 \text{ kPa}$$

It can be concluded that the present procedure for the anastomotic operation results in a decrease of the mechanical strength of the colonic segments under study of the order of only 35% in comparison to the intact specimens.

As a final step, a third series of tests was carried out with specimens obtained from non-healthy rats after having been subjected to colon anastomotic surgery. It was strange to observe that the scattering of the results was rather lower in this case and the application of the Chauvenet criterion yielded the exemption of only one test. A number of characteristic tests of this class experiments is shown in Figure 4D. The average value for the failure force, for this series of tests was determined equal to:

$$F_{fail}^{non-healthy} = 1.09 \text{ N} \pm 0.19 \text{ N}$$

In this case failure strength ranges between:

$$82 \text{ kPa} \leq \sigma_{fail}^{non-healthy} \leq 110 \text{ kPa}$$

The decrease of the mechanical strength compared to the intact specimens is of the order of about 50%, while if the comparison is carried out on the basis of the results of the control tests, is of the order of about 19%.

DISCUSSION

Wound leakage, the major concern for every surgeon performing intestinal anastomosis, is considered a multifactorial process, upon which many factors act, accelerating or inhibiting its metabolic pathway^[17,18]. Numerous clinical entities and metabolic abnormalities can alter the course of tissue repair. Amongst them diabetes mellitus, hypothyroidism, immunocompetence, infection and other diseases are proven to be detrimental to anastomotic healing, while other factors like the surgical technique, advanced age, malnutrition, obesity, inadequate perfusion and/or oxygenation are considered risk factors for impaired wound healing^[19-22].

Taking the 5th post-operative day as a crucial time point upon which anastomotic failure is mostly recognized in clinical practice, the authors tried to give a measure of the anastomotic strength by taking advantage of its mechanical behavior. While both bursting pressure and tensile strength are used to describe the mechanical properties of viscoelastic materials like the ones under study, the authors preferred to evaluate the second and correlate it to the healing of colonic anastomosis. This is because tensile strength appears to be a better standard to evaluate the biological aspects of healing. Tensile strength is an important determinant of anastomotic strength, in contrast to the bursting pressure, which can evaluate the overall anastomotic integrity, but may reflect healing less accurately.

The authors in this paper described not only a system for gripping the specimens, the load application, the data acquisition and storage system, but also a detailed view of the theoretical background behind the forces applied in the tissues under study, as well as the experimental difficulties of the direct tension experiment.

The values of the load as a function of the time [$P = P(t)$] and the values of the displacement of the moving plate of the loading frame also as a function of time [$\Delta s = \Delta s(t)$] were recorded, giving the load versus the displacement curve for each measurement and therefore providing the recorded discontinuities due to the anastomotic failure.

While the tests performed were used only for a preliminary series of measurements, since the number of specimens was relatively small, significant conclusions can be made regarding wound strength and tissue regeneration. The decrease of the axial force required causing mechanical failure from 2.09 N in case of the “intact” specimens to about 1.35 N for the control tests and to 1.09 N for the specimens from non-healthy rats is an excellent index of the quality of the anastomotic operation. Of course, a larger number of measurements need to be carried out, so as to provide a more rigid approach to tissue leakage, its quantitative expression through the tensile strength experiments, and its clinical correlations with pathological entities that delay wound healing or with factors that promote anastomotic integrity and repair.

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BASIC RESEARCH

Localization of ANP-synthesizing cells in rat stomach

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in the gastric mucosa. EC synthesize ANP. There is a close relationship between ANP-synthesizing cells and microvessel density in gastric mucosa of rats. The distribution density of ANP-synthesizing cells is largest in the gastric cardiac region.

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Key words: Atrial natriuretic peptide-synthesizing cells; Microvessel density; Close relationship; Gastric cardiac region

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Abstract

AIM: To study the morphological positive expression of atrial natriuretic peptide (ANP)-synthesizing cells and ultrastructural localization and the relationship between ANP-synthesizing cells and microvessel density in the stomach of rats and to analyze the distribution of the three histologically distinct regions of ANP-synthesizing cells.

METHODS: Using immunohistochemical techniques, we studied positive expression of ANP-synthesizing cells in rat stomach. A postembedding immunogold microscopy technique was used for ultrastructural localization of ANP-synthesizing cells. Microvessel density in the rat stomach was estimated using tannic acid-ferric chloride (TAFC) method staining. Distribution of ANP-synthesizing cells were studied in different regions of rat stomach histochemically.

RESULTS: Positive expression of ANP-synthesizing cells were localized in the gastric mucosa of rats. Localization of ANP-synthesizing cells identified them to be enterochromaffin cells (EC) by using a postembedding immunogold electron microscopy technique. EC cells were in the basal third of the cardiac mucosa region. ANP-synthesizing cells existed in different regions of rat stomach and its density was largest in the gastric cardiac region, and the distribution order of ANP-synthesizing cells in density was cardiac region, pyloric region and fundic region in mucosa layer. We have also found a close relationship between ANP-synthesizing cells and microvessel density in gastric mucosa of rats using TAFC staining.

CONCLUSION: ANP-synthesizing cells are expressed

INTRODUCTION

Since atrial natriuretic peptide (ANP) was isolated from atrium by de Bold *et al* in 1981^[1-3], brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), dendroaspis (DNP), micrurus natriuretic peptide (MNP), and ventricular natriuretic peptide (VNP) have been found in succession. They distribute not only in the heart but all over the body^[4-10]. ANP regulates a variety of physiological functions, including natriuresis, diuresis and vasodilation. Three types of natriuretic peptide receptors for ANP, BNP and CNP have been identified, for example, natriuretic peptide receptor type A (NPR-A), type B (NPR-B) and type C (NPR-C). NPR-A has guanylate cyclase activity and mediates the biological functions of ANP through the synthesis of cGMP^[2]. NPR-A preferentially binds ANP and BNP, but has a low affinity for CNP. Although ANP is synthesized primarily in the heart as a cardiac hormone, in fact ANP and its receptor are expressed in numerous extracardiac tissues, e.g., lung, thymus, gastrointestinal tract, suggesting a possible role as a regional regulator acting as an autocrine and/or paracrine regulatory peptide^[11,12]. Our previous study indicated that NPR existed in different regions of gastric mucosa and its density was the largest in rat gastric antrum, and NPR significantly inhibited spontaneous contraction of gastric smooth muscles in rats, guinea-pigs and humans^[13-16]. However, the distribution of ANP-synthesizing cells and the relationship between the distribution of ANP-synthesizing cells and microvessel density in gastric tissues have not been identified. It is also not clear about the ultrastructural localization of ANP-synthesizing cells in rat stomach. Therefore, in the present

study, the morphological distribution and ultrastructural localization of ANP-synthesizing cells were identified under postembedding immunoelectron microscopy. The relationship between distribution of ANP-synthesizing cells and microvessel density was investigated using histochemical techniques in rat stomach.

MATERIALS AND METHODS

Animals

Wistar rats (obtained from the Experimental Animal Center of Yanbian University College of Medicine) of either sex weighing 300-350 g were anaesthetized by a lethal dose of abdominal cavity injection of pentobarbital sodium (30 mg/kg), and the abdomen of each rat was opened along the midline. Because the rat stomach demonstrates significant regional differences in structure, we separated the stomach into three regions: the fundic or fundus, the cardiac, and the pyloric region or antrum (Figure 1).

Immunohistochemistry

Freshly excised atria and stomach were fixed in 40 g/L formaldehyde fixative and embedded in paraffin. Sections (5 μ m) were deparaffinated, rehydrated and incubated with 3 mL/L hydrogen peroxide in methanol for 15 min at room temperature to block endogenous peroxidase activity. After washing twice with phosphate-buffered saline (PBS) for 5 min, tissue sections were incubated at 37°C for 20 min with blocking solution. Sections were incubated at 37°C for 2 h with primary antibody: rabbit anti-rat ANP (sc-20158, Santa Cruz Biotechnology, Inc) 1:100. After washing twice with PBS (0.01 mol/L, pH7.4) for 10 min, tissue sections were incubated at 37°C for 30 min with biotin-anti-rabbit IgG. After washing two times in PBS for 5 min, the sections were incubated with streptavidin-HRP for 30 min. Then the sections were washed two times in PBS for 5 min, and they were incubated with metal-enhanced 3,3-diaminobenzidine solution for 15 min, then they were washed two times in distilled water and counterstained with hematoxylin. Negative control sections were incubated with normal rabbit serum instead of primary antibody. The positive staining for ANP-synthesizing cells was expressed as red brown granules, which were mainly located in cell cytoplasm under microscopy. At least 5 high-power ($\times 400$ field) fields were chosen randomly for cell counting. The ratio of the positive distribution of ANP-synthesizing cells was calculated by dividing the number of positive cells over the total number of cells, and was expressed as percentage, counted, analyzed under the CMIAS image analysis system (Beihang, China), and photomicrographed (Olympus PM-10AD).

Immunoelectron microscopy

Freshly excised stomach mucosal and atrial myocyte blocks of 1 mm³ were fixed in 100 mL/L paraform fixative, then dehydrated and embedded in Epon-812 resin. Sections (70 nm) were incubated with 100 mL/L hydrogen peroxide in methanol for 30 min at room temperature. After washing

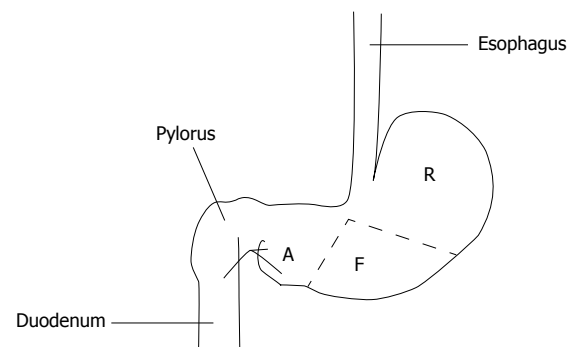


Figure 1 Different histological regions of rat stomach. R: Cardiac region or cardia; F: Fundic region or fundus; A: Pyloric region or antrum.

twice with distilled water for 10 min, tissue sections were incubated at room temperature for 1 h with blocking solution, then tissue sections were incubated at 4°C for 36 h with primary antibody: rabbit anti-rat ANP (sc-20158, Santa Cruz Biotechnology, Inc) 1:90. Negative control sections were incubated with normal rabbit serum instead of primary antibody. After washing twice with PBS for 5 min, tissue sections were incubated at room temperature for 1 h with protein A-10 nm colloidal gold labeled (Product Number P 6730, Sigma). After washing twice with distilled water for 5 min, sections were stained with urenyl acetate and lead citrate each for 5 min and were observed under a JEM-1200EX, 80 kv electron microscope (JEOL, Japan).

Histochemistry

Freshly excised rat stomach tissues were fixed with paraformaldehyde fixative (formaldehyde: potassium = 1:4) for 36 h at 4°C. The tissues were then fixed in 3% potassium bichromate fixative for 12 h at room temperature, and embedded in paraffin. Histochemical staining for ANP-synthesizing cells were performed by the chromaffin staining method. After washing twice with distilled water for 20 min, the tissues were stained at room temperature for 20 min with 10 g/L toluidine solution, and after washing twice with distilled water for 10 min, the sections were stained at room temperature for 15 min with 1% saffron solution. Chromaffin staining was made for enterochromaffin cells. The positive cells were expressed as brown granules, which were mainly located in cell cytoplasm under microscopy. At least 5 high-power ($\times 400$ field) fields were chosen randomly for cell observation. The area density of the distribution of positive cells was calculated under light microscopy (Olympus BH-2, Japan) and with CMIAS image analysis system (Beihang, China).

Tannic acid-ferric chloride method

The gastric microvessels in density and distribution were investigated in different regions of stomach. After the experimental rats (Wistar rats) were perfused with 20 g/L compound fixative tannic acid solution, the stomachs were taken out and cut into frozen sections, then these sections were immersed in 20 g/L ferric chloride solution at room temperature for 20 min to reveal the microvessels. The blood vessels were revealed distinctively by TA-Fe staining

method, and observed under the light microscope, the density and distribution of the vessels were measured and analyzed by CMIAS image analysis system. Density and distribution of microvessels in rat stomach was determined according to the KONG Xiang-yu *et al* method^[13], and was photomicrographed (Olympus PM-10AD, Japan).

Statistical analysis

Data were expressed as mean \pm SD. The two-tailed χ^2 test was used to examine the correlation between ANP-synthesizing cells and microvessel density. Statistical significance was estimated by *t* test. Differences were considered significant when $P < 0.05$. All the calculations were performed using SPSS11.0.

RESULTS

Expression of ANP-synthesizing cells in rat stomach

Immunohistochemical positive expressions of ANP-synthesizing cells were exhibited in paraffin sections of atria and stomach. As a positive control, ANP-synthesizing cells showed intense positive expression in atrial myocytes cytoplasm (as red brown granules, Figure 2A). ANP-synthesizing cells also were positively expressed in gastric mucosa and the positive granules localized to cytoplasm in the basal portion of cardiac region glands (Figure 2B and C). In negative controls, complete absence of positive staining for ANP-synthesizing cells was observed when normal rabbit serum was substituted for primary antiserum (Figure 2D). The morphological shape of the individual ANP-synthesizing cells was variable, exhibiting round, pyramidal, flask shapes, etc. The general epithelial morphology of these cells was typically endocrine in appearance, and most immunoreactivity was localized to the basal portion of the stomach of rats. Negative staining for ANP-synthesizing cells was detected in the lamina propria, submucosa, and smooth muscle. Expression of ANP-synthesizing cells was exhibited in different regions, and its density was the largest in gastric cardiac region, and the density order of ANP-synthesizing cells was cardiac region (cardia) > pyloric region (antrum) > fundic region (fundus) in mucosal layer.

Identification of ANP-synthesizing cells in rat stomach

The gastric mucosa was cut into ultra-thin sections, and situs ultrastructural detection of ANP-synthesizing cells was carried out using a post-embedding immunogold labeling technique under electron microscope. The localization of immunogold labeling was displayed in the endocrine granule of the enterochromaffin (EC) cell of DNES (disperse or diffuse neuroendocrine system, DNES) in gastric gland of rat (Figure 3A). These results indicate that the EC cell synthesizes and secretes ANP in gastric mucosa of rat. In negative control, complete absence of positive staining was observed when normal rabbit serum was substituted for anti-ANP antiserum (Figure 3B and C).

Distribution of ANP-synthesizing cells in rat gastric mucosa

The distribution of ANP-synthesizing cells (enterochromaffin cell, EC cells) in the different regions of gastric mucosa was detected using a histochemical

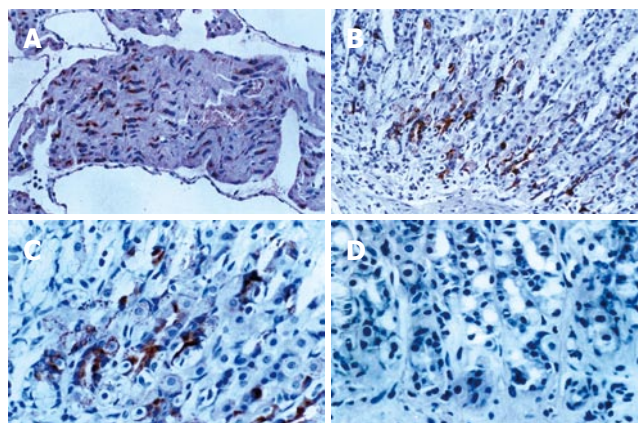


Figure 2 A: As a positive control, atrial myocytes show intense positive cytoplasmic staining for ANP (IHC \times 200); B: Positive staining for ANP is localized to cytoplasm of mucosal cells in cardiac glands (IHC \times 200); C: Positive staining for ANP is localized to cytoplasm of mucosal cells in cardiac glands (IHC \times 400); D: As a negative control, complete absence of staining is observed when normal rabbit serum is substituted for ANP (IHC \times 400).

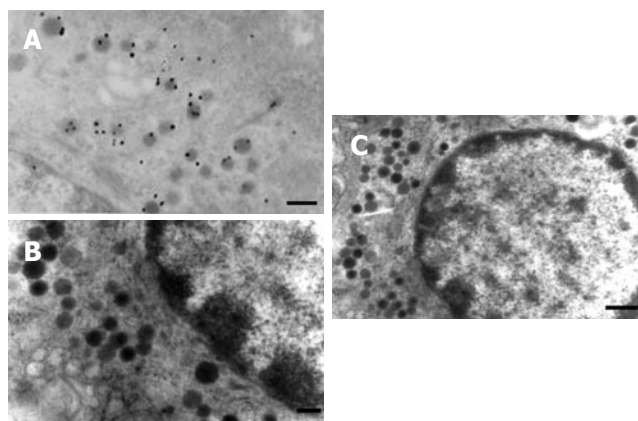


Figure 3 A: localization of immunogold labeled in the endocrine granule of the enterochromaffin cell (TEM \times 20 000, bar = 200 nm); B: Negative control, complete absence of immunogold labeled in the endocrine granule of enterochromaffin cells when normal rabbit serum was substituted for anti-ANP antiserum (TEM \times 20 000, Bar = 200 nm); C: Normal enterochromaffin cells in gastric mucosa (TEM \times 15 000, Bar = 500 nm).

technique in rats. It has been identified that EC cell is only a chromaffin cell in the rat gastric mucosa. Consecutive serial sections were stained for chromaffin and the chromaffin granules (brown granules) were localized in EC cell cytoplasm. Negative staining for chromaffin granules were detected in the lamina propria, submucosa, smooth muscle and in negative control. The distribution of EC cells in gastric mucosa was further examined using chromaffin staining. There were three histologically distinct regions (cardia, fundus and antrum) in the distribution of EC cells in rats. EC cells existed in mucosal layer, and its density was the largest in gastric cardia, and the density order of EC cells (mean \pm SD) was cardiac region > pyloric region > fundic region in mucosal layer (Figure 4, $n = 18$).

Relationship between ANP-synthesizing cells and microvessel density

The microvessels were stained successfully with tannic acid-ferric chloride^[13,14]. Microvessel of gastric mucous

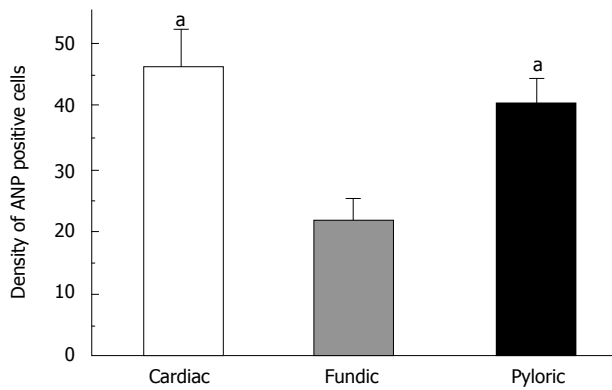


Figure 4 The distribution of ANP synthesizing cells in different regions of stomach in rats. Mean ± SD. ^a $P < 0.05$ vs fundic region.

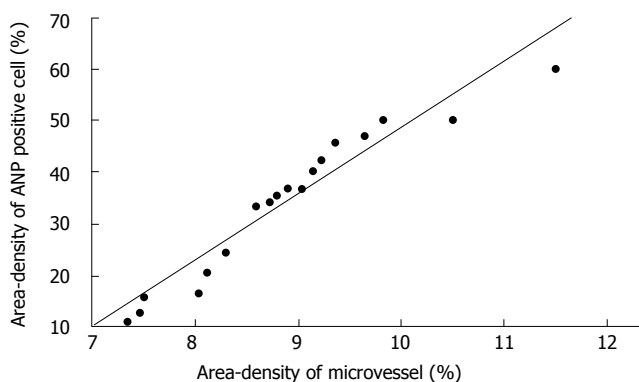
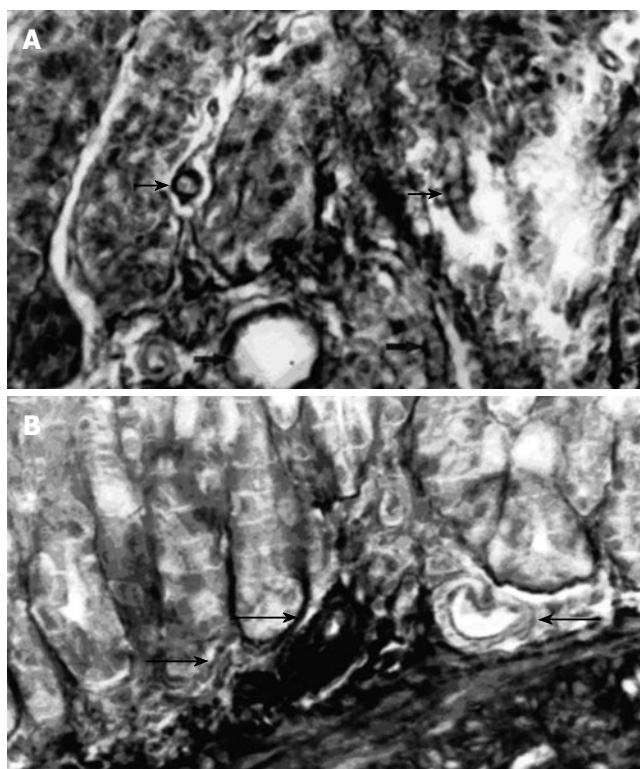


Figure 5 **A:** Microvessels of gastric mucosa were in wriggled way and cut into different cross-sections, it could be clearly observed in distinct three-dimensions (arrow) (TA-Fe × 400); **B:** Some branch arteries from the large vessels run into the basal glands of the gastric mucosa (arrow) (TA-Fe × 400); **C:** There is positive significant relationship between the positive rate (%) of ANP-synthesizing cells and microvessel density (%) in cardiac region mucosa of rats ($r = 0.53$, $P < 0.05$, $n = 18$).

were in wriggled way and cut into different cross-sections, it could be clearly observed in distinct three-dimensions (Figure 5A). Some microvessels could be found scattering among antral mucosa, some branch arteries from the large vessels ran into the basal glands of the gastric mucosa (Figure 5B). The density of microvessels varies from position to position, the more basal glands were found, the more microvessel were distributed in rat gastric mucosa. In order to study the relationship between the positive rate of ANP-synthesizing cells and microvessel density in rat gastric mucosa, the data were analyzed by statistical analysis system of SPSS11.0. There was a positive significant relationship between the positive rate of ANP-synthesizing cells and microvessel density in antral mucosa of rats ($r = 0.53$, $P < 0.05$, Figure 5C).

DISCUSSION

In the present study, the ANP-expressing myoendocrine cells are most concentrated in the right atrium, to a lesser extent in the left atrium, and almost absent in the left ventricle^[15,16]. We have demonstrated that morphological distribution and ultrastructural localization of ANP-synthesizing cells in rat stomach. ANP may have tissue-specific functions within the stomach. Gower *et al*^[17-19] and Vuolteenaho *et al*^[20-24] earlier found that the rat antrum contains ANP-synthesizing cells coupled with our present results that similar immunostaining patterns are produced when antibodies are directed to antral mucosal cells, however we have also found positive cells in cardiac and fundus. EC cells are an abundant type of enteroendocrine cells that contain serotonin and occur throughout the gastrointestinal tract^[25-28]. On the basis of differences in the ultrastructural appearance of the secretory granules, it has been suggested that EC cells are comprised of several subpopulations and store different peptides. Three types of EC cells for EC₁, EC₂, and EC_n have been identified. EC cells have two types of open-type enterochromaffin cell and close-type enterochromaffin cell. Open-type enterochromaffin cells have a large basolateral compartment in contact with the basal lamina and a narrow apical process that allows access to the lumen. Results from our present immunohistochemical studies demonstrate that at least some of the ANP-synthesizing cells in the gastric mucosa are exposed to both basal lamina and lumen, and under immunoelectron microscopy they were identified as EC cells that synthesized ANP.

Guo HS *et al*^[29] using a radioautograph technique, detected the distribution of NPR (NPR-A) in different regions of rat stomach. NPR-A existed in both the mucosal layer and muscle layer, and the distribution order of NPR-A in density being antrum > body > fundus in muscle layer. Rambotti *et al*^[30] found that NPR-A extensively distributed in many tissues, for example, the bladder of the toad, bullfrog brain, in fetal ovine pulmonary vascular and in the porcine coronary. The present study confirmed that NPR-A also existed in the stomach of rat and the density was largest in gastric antrum in rats, demonstrating the presence of NPR-A transcripts in the extracts of gastric fundus and natriuretic peptide-induced cGMP production

localized to the parietal, mucus secreting cells in the fundus, and pyloric glands, as well as gastric smooth muscle cells^[29]. Because ANP is known to stimulate gastric acid secretion and relaxes gastric smooth muscle, the effect of atrial natriuretic factor (ANF) on exocrine pancreatic secretion and the possible receptors and pathways involved were studied *in vivo*^[29,31]. These findings suggest that these effects of ANP may be direct. Rambotti *et al*^[31] demonstrated the presence of ANP-induced guanylate cyclase activity on both apical and basolateral surfaces of mucosal cells within the pyloric glands of rat stomach, ANP released locally into the gastric lumen could target luminally directed receptors, suggesting that ANP may help control a “negative feedback” system within the stomach of increasing acid secretion and simultaneously enhance mucus production to protect the lining of the stomach from the effects of acid. This would provide for a regulatory mechanism to ensure that the acid produced after a meal does not injure the mucosal surface of the stomach. Previous studies also indicated that vagus nerve regulated many functions of NP, for example, ANP promoted gastric acid secretion^[32], ANP and endothelin-1 (ET-1) might prevent renal dysfunction during the progression of congestive heart failure (CHF) through the cGMP pathway in dogs^[33,34], ANP can reduce the pre-load and after-load in normal and failing hearts^[35-37]. ANP each inhibited the growth of the human pancreatic adenocarcinomas *in vivo* and three of the four peptide hormonesed the volume of the tumors (up to 49%)^[10,38,39]. The intestinal tract is a target organ for ANP, characterized by various biologic activities, immunoreactivity, as well as specific binding sites for ANP^[40,41].

In conclusion, our result demonstrated that ANP-synthesizing cells exist in gastric mucosa of rats, and its density was largest in gastric cardiac region, and the distribution order of ANP-synthesizing cells in density was cardiac region > antrum > fundic region in mucosa layer. This result shows that it is the EC cells in the gastric mucosa that synthesize ANP. This basolateral plasma membrane is juxtaposed to microvessel *via* ANP which could enter the circulation from the stomach. The density of microvessel varies from position to position at the EC cell concentrating area, and the relationship between distribution of ANP-synthesizing cells and microvessel density was investigated in the stomach of rats.

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CLINICAL RESEARCH

Multifactorial analysis of risk factors for reduced bone mineral density in patients with Crohn's disease

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bone loss. However, less than half of the reduction in BMD can be attributed to risk factors such as corticosteroid use and low BMI and therefore remains unexplained.

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Key words: Crohn's disease; Osteoporosis; Osteopenia; Bone mineral density

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Abstract

AIM: To determine the prevalence of osteoporosis in a cohort of patients with Crohn's disease (CD) and to identify the relative significance of risk factors for osteoporosis.

METHODS: Two hundred and fifty-eight unselected patients (92 M, 166 F) with CD were studied. Bone mineral density (BMD) was measured at the lumbar spine and hip by dual X-ray absorptiometry. Bone formation was assessed by measuring bone specific alkaline phosphatase (BSAP) and bone resorption by measuring urinary excretion of deoxypyridinoline (DPD) and N-telopeptide (NTX).

RESULTS: Between 11.6%-13.6% patients were osteoporotic (T score < -2.5) at the lumbar spine and/or hip. NTX levels were significantly higher in the patients with osteoporosis ($P < 0.05$) but BSAP and DPD levels were not significantly different. Independent risk factors for osteoporosis at either the lumbar spine or hip were a low body mass index ($P < 0.001$), increasing corticosteroid use ($P < 0.005$), and male sex ($P < 0.01$). These factors combined accounted for 23% and 37% of the reduction in BMD at the lumbar spine and hip respectively.

CONCLUSION: Our results confirm that osteoporosis is common in patients with CD and suggest that increased bone resorption is the mechanism responsible for the

INTRODUCTION

Osteoporosis is now recognised as a common complication of inflammatory bowel disease (IBD) and in particular Crohn's disease (CD). Estimates of prevalence vary but those studies employing the World Health Organisation^[1] diagnostic criteria (a bone density 2.5 or more standard deviation units below the mean value for young adults) report rates of 13%-42%^[2-4]. The pathogenesis of osteoporosis in patients with CD is likely to be multifactorial. Most, but not all studies, have found an association with current or cumulative corticosteroid use^[5-11] but other factors such as disease duration^[3,4,12] low body weight or body mass index^[4,10,13] calcium and vitamin D deficiency^[12,14] small bowel involvement or resection^[10] smoking^[13] gender and increasing age^[13] have been implicated. At the molecular level *in vitro* studies have demonstrated that serum from children with CD can inhibit bone formation suggesting that pro-inflammatory cytokines such as IL-6 may be involved^[16].

The mechanism of bone loss is poorly understood. Normal bone in healthy adults is in a state of equilibrium, the rate of osteoblastic bone formation equaling the rate of bone resorption by osteoclasts. Biochemical markers of bone turnover such as deoxypyridinoline (DPD) and cross-linked N-telopeptides of type 1 collagen (Ntx), both markers of bone resorption, and osteocalcin and bone specific alkaline phosphatase (BSAP), markers of bone formation, are now available and have been used in several studies examining the possible mechanisms of bone loss in patients with IBD. Consistent findings are raised levels

of either DPD or Ntx^[2,4,12,17,18] suggesting increase in bone resorption, although there is also evidence that bone formation is reduced^[7].

The aim of this cross sectional study was to determine the prevalence of osteoporosis in an unselected group of patients with CD and to identify the relative importance of possible risk factors and the mechanism of bone loss.

MATERIALS AND METHODS

Patients were recruited from the IBD register at the Freeman Hospital and additionally from the Royal Victoria Infirmary, also in Newcastle-upon-Tyne, and the Queen Elizabeth Hospital, Gateshead. Patients were under the care of either surgical or medical gastroenterologists and all fulfilled at least two of four diagnostic criteria (histological, radiographic, endoscopic and surgical)^[19]. Patients were contacted by letter inviting them to participate in the study and were then seen at the Freeman Hospital by either SB or NT. Those who did not attend were sent one further letter reminding them to do so. We included patients aged between 25 and 70 years only and excluded women who were pregnant or planning a pregnancy because of the potential risk from exposure to ionizing radiation during bone densitometry.

A questionnaire was completed with the patient detailing age, tobacco and alcohol consumption, fracture history, and in women, reproductive and menstrual history. Details of duration and site of disease, corticosteroid use (expressed as number of months on corticosteroids), relevant surgical history and drug history were obtained at interview and by careful scrutiny of the medical notes. Height and weight was measured immediately prior to bone densitometry and these figures were used to calculate body mass index [weight/height², (kg/m²)]. The study was approved by the Newcastle-upon-Tyne Joint Ethics Committee.

Bone mineral density measurements

Bone mineral density (BMD) was measured at the lumbar spine (L1-L4) and left hip (total hip) by dual X-ray absorptiometry (Hologic Inc. QDR 2000, Waltham, MA.). The coefficient of variation (CV) is 0.7% at the lumbar spine and 1.0% at the hip^[20]. BMD results are expressed as an areal density in g/cm², but have been compared with the manufacturer's mean value for young adults and for normal people of the same age and sex to give T scores and Z scores respectively. The T score is the number of standard deviation units above or below the mean value for young adults of the same sex, whilst the Z score is the number of standard deviation units above or below the age related mean value. The WHO has defined osteopenia as a T score of < -1 but > -2.5, whilst osteoporosis is defined as a T score of -2.5 or lower. These diagnostic criteria are not necessarily thresholds for intervention, particularly for patients on oral corticosteroids, where a higher T score of -1.5 would be more appropriate^[21].

Biochemical measurements

Serum and urine samples were taken between 2 pm

and 4 pm. The serum was immediately centrifuged and all samples were stored at -30°C. Serum calcium and phosphate levels were measured using standard methods on an Olympus 600 automated system, (interassay CVs for calcium and phosphate are < 1% and < 2% respectively).

Bone resorption was assessed by measurement of the urinary excretion of free DPD and Ntx with values expressed as a fraction of urinary creatinine excretion. Free DPD was measured by competitive immunoassay (Chiron Diagnostics Corporation, MA) (interassay CV < 8%). The reference range (manufacturer's data), is 3.0-7.4 nmol DPD/mmol creatinine for females aged 25-44 years and 2.3-5.4 nmol DPD/mmol creatinine for males aged 25-55 years. Ntx was measured by enzyme-linked immunoabsorbant assay ("Osteomark", Ostex International, Seattle, WA) with results expressed as bone collagen equivalents (BCE). The reference ranges for men and women (manufacturer's data) are 3-51 and 5-65 nm/mmol creatinine respectively (interassay CVs < 9%). Bone formation was assessed by measurement of serum BSAP using enzyme-linked immunoabsorbant assay (Metra BioSystems, Southampton, UK) (interassay CV < 6%).

Patients with a T score of -1.5 or less at either the lumbar spine were investigated further to look for other secondary causes of osteoporosis, such as vitamin D deficiency with secondary hyperparathyroidism, thyroid disease and, in men, hypogonadism.

Intact parathyroid hormone (PTH) levels, reference range 12-72 ng/L, were measured by immunometric assay (Immulite Intact PTH, Diagnostics Products Corporation, Los Angeles, CA.) (interassay CV < 5%). Vitamin D levels (25 OH Cholecalciferol and 25 OH Ergocalciferol), normal range 10-50 nmol/L, were assayed by high performance liquid chromatography performed at the Department of Clinical Biochemistry, Royal Victoria Infirmary, Newcastle-upon-Tyne (interassay CV < 12%). Thyroid stimulating hormone (TSH), reference range 0.3-4.1 Mu/L, was measured with a two site immunoassay using direct chemiluminometric detection and monoclonal/polyclonal antibodies (interassay CV < 4.0%). Testosterone, reference range 9-20 nmol/L, was measured with a competitive immunoassay using direct chemiluminometric detection and a polyclonal antibody (interassay CV < 5.2%) and Sex Hormone Binding Globulin (SHBG) was measured with a non-competitive immunoradiometric assay (interassay CV < 5%). The free androgen index (FAI), reference range > 3 nmol/nmol, was calculated after dividing the total serum testosterone by the SHBG level.

Statistical analysis

The results are expressed as means \pm SD. Comparison between group means was analysed using Student's unpaired *t*-test or, where data was not normally distributed, the Mann-Whitney *U*-test. Multiple regression analysis was performed to determine independent risk factors for osteoporosis and the adjusted χ^2 value was calculated to determine the proportion of the variability in BMD attributable to these factors. The chi-squared test was used to compare incidences. A *P* value of < 0.05 was taken to be statistically significant. Analyses were made with

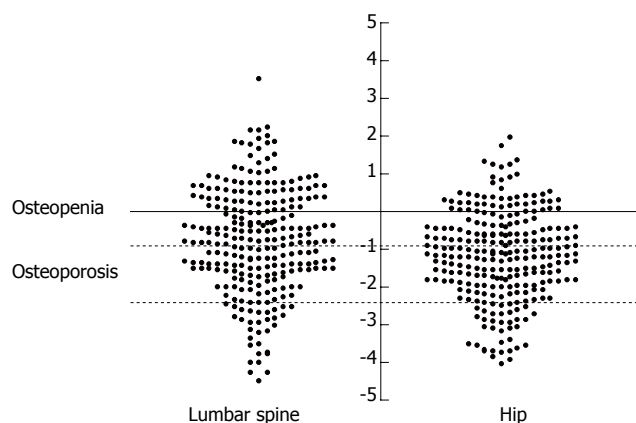


Figure 1 Scatterplot of T score values at the lumbar spine and hip. Dotted lines represent thresholds for diagnosis of osteopenia and osteoporosis.

GraphPad Prism (GraphPad Software Inc) and Minitab (Version 12.1, Minitab Inc).

RESULTS

Two hundred and sixty five patients responded to the correspondence inviting them to participate in the study, 7 of whom subsequently declined a DXA scan and were excluded from the study. The remaining 258 patients who completed the study comprised 92 men and 166 women. One patient was of Asian descent, the remainder were white. Table 1 summarizes the clinical characteristics of the group.

Fractures

Twenty-eight patients (10.9%) reported a low trauma fracture in adulthood, 12 of whom had osteoporosis. The fractures comprised wrist^[9], forearm^[5] and vertebral^[3] fractures, and 2 each of metatarsal, rib and ankle fractures, 1 patella fracture and 1 femoral fracture. Two patients had more than 1 fracture and in 5 cases the fracture site was not documented.

Site of disease

One hundred and ninety two (74.4%) had small bowel disease, of which 70 (27.1%) had small bowel involvement alone and 88 (34.1%) had small bowel involvement with colonic disease. Forty-nine (18.9%) had isolated colonic disease. At least one partial small bowel resection had been performed in 135 (52.3%) of the study group, whilst 92 (35.7%) had undergone partial or total resection of their large bowel.

Drug treatment

Seventy-seven patients (29.8%) were currently taking oral corticosteroids and 152 (58.9%) had previously taken corticosteroids to treat complications of their Crohn's disease. Forty-five patients (17.4%) were currently taking bone active treatment (calcium supplementation with or without vitamin D, bisphosphonates or hormone replacement therapy).

Table 1 Patient characteristics (means \pm SD)

<i>n</i>	258 (166 F, 92 M)
Age (yr)	44.5 (\pm 11.5)
Postmenopausal women	67 (25.9%)
Fractures	28 (10.8%)
BMI (kg/m^2)	24.7 (\pm 4.6)
Disease duration (yr)	14.3 (\pm 9.5)
Small bowel involvement	192 (74.4%)
Small bowel resection	135 (52.3%)
Large bowel involvement	173 (67.1%)
Current CS use	77 (29.8%)
Previous CS use	152 (58.9%)
Duration CS use (mo)	51.0 (\pm 68.4)
Current smokers	83 (32.2%)

CS: Corticosteroids.

Table 2 Bone mineral density measurements (means \pm SD)

Site	BMD (g/cm^2)	Z score	T score	Osteopenia	Osteoporosis
Lumbar spine	0.975 \pm 0.15	-0.308 \pm 1.39	-0.795 \pm 1.39	77 (29.8%)	30 (11.6%)
Total hip	0.860 \pm 0.15	-0.675 \pm 1.19	-1.181 \pm 1.21	67 (25.9%)	35 (13.6%)

Osteopenia defined as a T score of < -1 but > -2.5 ; Osteoporosis defined as a T score of < -2.5 .

Tobacco and alcohol consumption

Eighty-three patients (32.2%) were current cigarette smokers and 78 (30.2%) previous smokers. The mean alcohol intake was 8.8 units/week among the 160 patients who drank alcohol.

Bone mineral density measurements

Measurements at the lumbar spine were available in 257 patients, one was excluded because of the presence of surgical metal rods. Measurements at the hip were available in all 258 patients. Details of the mean BMD measurements, mean Z and T scores and numbers with osteopenia or osteoporosis are presented in Table 2 and Figure 1. Employing the WHO definitions for osteoporosis 18 (6.9%) were osteoporotic at both the lumbar spine and hip. Considering each site separately, 30 (11.6%) patients were osteoporotic at the lumbar spine (16 men, 14 women) and 35 (13.6%) were osteoporotic at the hip (15 men, 20 women) whilst a further 77 (29.8%) and 67 (25.9%) fulfilled the criteria for osteopenia at the lumbar spine and hip respectively.

Risk factors

Table 3 shows the characteristics of those patients with and without osteoporosis. At the lumbar spine patients with osteoporosis had a significantly lower BMI and had taken corticosteroids for longer, there was a trend towards longer disease duration although this did not achieve statistical significance. Patients with osteoporosis at the hip were also likely to have a lower BMI, a longer disease duration and have taken corticosteroids for longer. Gender, small bowel resection and current tobacco use were not

Table 3 Comparison of those patients with and without osteoporosis and the presence of risk factors (mean \pm SD)

Risk factor	Osteoporosis at lumbar spine and/or hip (<i>n</i> = 47)	Osteopenia/normal bone mineral density (<i>n</i> = 211)	<i>P</i>
BMI (kg/m ²)	22.3 \pm 0.6	25.2 \pm 0.3	< 0.0001
Corticosteroid use (mo)	92.3 \pm 16.7	34.9 \pm 3.2	< 0.0001
Disease duration (yr)	18.9 \pm 1.5	13.2 \pm 0.6	< 0.005
Age (yr)	46.8 \pm 11.4	44.1 \pm 11.5	NS
Small bowel resection (Y/N)	10/37	105/106	NS
Current smokers (Y/N)	11/36	72/139	NS
Gender (M/F)	22/25	69/142	NS

Analysis was made using Student's unpaired *t*-test or the chi-squared test where appropriate; NS: not significant.

significant factors at either site. One hundred and fifteen patients (44.6%) had T scores < -1.5 at the lumbar spine and/or hip respectively. The T score within individual patients was significantly lower at the hip compared with the lumbar spine (-0.80 ± 1.4 *vs* -1.19 ± 1.2 , $P < 0.0001$).

Postmenopausal women had significantly lower T scores at the lumbar spine and hip compared to the rest of the study group (-1.30 ± 0.16 *vs* -0.63 ± 0.10 and -1.5 ± 0.14 *vs* -1.09 ± 0.09 respectively, $P < 0.0001$). This group accounted for 11/29 patients with osteoporosis at the spine and 12/34 at the hip.

Multivariate analysis was used to determine independent predictors for the presence of osteoporosis. Variables included in the analysis were age, sex, disease duration, BMI, length of corticosteroid use and small bowel resection. Of these, decreasing BMI ($P < 0.001$), length of corticosteroid use ($P = 0.003$) and male sex ($P = 0.009$) were the significant predictors at the lumbar spine whilst at the hip decreasing BMI ($P < 0.001$), number of months treatment with corticosteroids ($P < 0.001$), increasing age ($P = 0.003$) and male sex ($P = 0.005$) were significant. The adjusted r^2 value was calculated using sex, age, sex-age interaction, corticosteroid use, BMI, disease duration and small bowel resection (Yes/No) as variables. These factors accounted for 22.9% and 37.2% in the variability in BMD at the lumbar spine and hip respectively.

Biochemical measurements

The mean levels of total calcium and phosphate were normal (Table 4). There was no difference in the means between those with and those without osteoporosis. Patients with a T score < -1.5 at either site ($n = 115$) were investigated further and in these patients mean levels of TSH and 25 OHD were normal (Table 5). Five patients had abnormal thyroid function tests. Two patients had low TSH levels, one of these patients was on thyroid replacement therapy. Of the three with above normal TSH levels, none had abnormal tri-iodothyronine or thyroxine levels. Two patients had biochemically low 25 OHD levels (defined as < 10 mol/L) but neither was associated with a secondary hyperparathyroidism or with small bowel resection. Mean PTH levels were normal. PTH levels were raised (defined as twice the upper limit

Table 4 Biochemistry and markers of bone formation and resorption in patients with osteoporosis at either the lumbar spine and/or hip compared with those patients with osteopenia or normal bone density (mean \pm SD)

Biochemistry (normal range)	Osteoporosis at lumbar spine and/or hip (<i>n</i> = 47)	Osteopenia/normal bone mineral density (<i>n</i> = 211)	<i>P</i>
Total calcium (2.12-2.55 mmol/L)	2.30 \pm 0.03	2.31 \pm 0.01	NS
Phosphate (0.65-1.3 mmol/L)	1.11 \pm 0.03	1.11 \pm 0.03	NS
BSAP (11.6-30.6 μ g/L)	19.7 \pm 1.4	18.6 \pm 0.6	NS
DPD/creat (2.3-7.4 nmol/mmol)	5.8 \pm 1.0	5.6 \pm 0.4	NS
Ntx/creat (3.0-65.0 BCE)	63.7 \pm 9.5	40.6 \pm 3.6	$P < 0.05$

BSAP: Bone specific alkaline phosphatase; DPD: Deoxypyridinoline; Ntx: N-telopeptides of type 1 collagen; BCE: Bone collagen equivalents; NS: not significant.

Table 5 Results of further bone biochemistry carried out in patients with a T score of -1.5 or less at either the lumbar spine and/or hip ($n = 115$) (mean \pm SD)

Biochemistry (normal range)	T score < -1.5 at lumbar spine and/or hip (<i>n</i> = 115)
TSH (0.3-4.1 Mu/L)	1.7 \pm 1.1
25 OHD (10-75 nmol/L)	45.1 \pm 22.3
PTH (10-72 ng/L)	46.9 \pm 62.7
¹ Testosterone (9-20 nmol/L)	13.5 \pm 6.7
² FAI (> 0.3 nmol/nmol)	0.4 \pm 0.1

¹Testosterone and SHBG only measured in men ($n = 46$). ²Free Androgen Index (Testosterone/SHBG).

of normal) in 4 patients (range 153-613 ng/L) including one patient with severe nutritional deficiency (BMI < 19, anaemia, hypoalbuminaemia) and severe osteoporosis who nevertheless had normal 25 OHD levels because of treatment with vitamin D metabolites prior to inclusion into the study. Three men (6.5%) were hypogonadal (FAI < 3 nmol/nmol).

Markers of bone formation and bone resorption

Mean levels of BSAP and DPD were normal, there was no significant difference between those with and without osteoporosis (Table 4). Ntx levels, although also within the normal range, were significantly higher in patients with osteoporosis.

DISCUSSION

We have shown that between 11%-13% of a large group of unselected patients with CD are osteoporotic. A low BMI, increasing exposure to corticosteroids and male sex appear to be the main predictors of osteoporosis which, however, only explain between 23%-37% of the variability in BMD described. Biochemical abnormalities such as 25 OHD deficiency and secondary hyperparathyroidism,

thyroid disease and male hypogonadism are unusual but our results suggest that increased bone resorption, assessed by measuring urinary excretion of DPD and Ntx, may be the mechanism responsible for the accelerated bone loss.

As far as we are aware this is the largest and most complete study to date of the prevalence of osteoporosis in patients with CD. Our group of patients are unselected and we consider a reasonable representation of the larger population with CD in terms of age and sex composition, duration and site of disease and disease management. Between 11%-13% of our patients are osteoporotic. This is perhaps lower than we expected given the unselected nature of our patient group, although broadly in keeping with other studies using the same diagnostic criteria. However, there is considerable discrepancy among prevalence rates reported. This may be accounted for by differences in the site and the methods used to measure BMD and in the patient groups studied, some for example including those not only with CD but also ulcerative colitis (UC), where there is evidence that osteoporosis is less common^[9,22]. Other groups have studied patients with CD only and have selected further to exclude for example those currently taking corticosteroids or at risk of metabolic bone disease^[2].

We found that the BMD at the hip was significantly lower compared with the lumbar spine. This has previously been reported in other studies of patients with IBD^[2-4,8,23] and is also the pattern of bone loss seen in other chronic inflammatory conditions such as rheumatoid arthritis. It may reflect a degree of degenerative change at the lumbar spine in some of the older patients which would spuriously elevate the density at that site, however we attempted to minimise this effect by excluding patients over the age of 70.

The clinical significance of osteoporosis is the increased susceptibility to fracture and the resulting morbidity and mortality. Eleven percent of our patients reported low trauma fractures, mostly of the upper limb. We did not perform radiography in our patients and, as many vertebral fractures are asymptomatic, the true prevalence is likely to be higher. Relatively few studies have examined fracture incidence and prevalence in adult patients with CD. In retrospective studies, fracture prevalence, calculated from plain radiography of symptomatic areas or self reported fracture, varies between 7% and 27%^[7,9,12]. Vestergaard *et al*, in a study of over 800 patients with IBD^[24] conducted *via* a postal questionnaire, reported an increased relative risk of low trauma fractures in women with CD of 2.5. The increase in fracture risk was seen mostly in premenopausal women but men with CD or patients of either sex with UC were not at increased risk of fracture. The authors speculated that their findings were due to a number of factors including the increased use of continuous corticosteroids and more severe systemic inflammation in CD compared to UC, and possible hypogonadism in the female patients.

A low BMI, increasing corticosteroid use, male sex and increasing age were independent risk factors in predicting those with osteoporosis although these factors together only accounted for between 20%-40% of the variability in BMD. Low body weight or a low BMI have been reported

in other studies of patients with IBD to be significant risk factors for osteoporosis, particularly at the peripheral sites^[4-6,9-11]. Within the context of IBD a low BMI may merely reflect severe inflammatory disease and/or malabsorption although there is also good evidence that low body mass *per se* is a significant factor in determining BMD^[25,26].

In our study male sex was a significant independent risk factor for osteoporosis at both sites. Most groups have reported no significant differences between the sexes and only one study has looked specifically at men with IBD. Robinson *et al* studied the hormone profile of 48 men with CD^[27] and found biochemical evidence of hypogonadism in only 3 patients, 2 of whom had osteopenia. Three of our male patients (6.5%) with reduced BMD were hypogonadal. This is lower than we might expect given the number of our patients taking corticosteroids which might lead to suppression of gonadotrophin, testosterone and SHBG levels.

The role of corticosteroids in the pathogenesis of osteoporosis in patients with IBD is complex. Whilst some studies have shown a clear relationship between lifetime corticosteroid dose and vertebral fracture rate^[5] or low BMD other studies have suggested that BMD is unrelated to corticosteroid use^[2,3,6,10]. There have been several prospective studies examining the role of corticosteroids in the rate of bone loss^[22,28,29]. The largest of these^[28], a longitudinal study of over 100 patients with IBD and bowel resection who were followed up for a mean period of over 5 years found no relationship between bone loss and corticosteroid use. The conflicting results are probably a reflection of the heterogeneity of patient groups studied and the complex relationship between disease severity, systemic inflammation and treatment with corticosteroids.

We found a very low prevalence of biochemical abnormalities in our patients and in particular no evidence of 25 OHD deficiency and secondary hyperparathyroidism or of a correlation with small bowel resection. The relationship between 25 OHD levels and BMD in patients with IBD is not clear cut. Andreassen^[14] reporting one of the higher prevalences of 25 OHD deficiency (44%) in a study of 115 patients with CD nevertheless found that this, in combination with PTH levels, only accounted for 4% of the variation in BMD. Croucher^[30] described the histomorphometric findings in a study of 19 patients with IBD, all of whom had osteoporosis and 16 of whom had undergone bowel resection. Despite only one patient having a low 25 OHD level, there was evidence of a mild mineralisation defect in the patient group compared with the control group, although none had the classical changes associated with osteomalacia.

Biochemical markers of bone formation and resorption are now widely available and have been used to predict bone loss^[31,32] and response to treatment of osteoporosis with bisphosphonates and hormone replacement therapy (HRT) in postmenopausal women^[33,34]. Several studies have now used these markers in exploring the mechanisms of bone loss in patients with IBD. Most studies have demonstrated an increase in bone resorption without a corresponding increase in bone formation^[2,4,12,17,18]. Our results, which show that patients with osteoporosis

have significantly higher levels of Ntx (but not DPD), would support the results of these studies. It should be noted however that the mean value, even in patients with osteoporosis, was within the normal range and their role in predicting those patients with IBD at risk of losing bone is not clear. Pollak *et al* has reported that increased levels of Ntx predicted rates of bone loss in a prospective study of 36 patients with IBD over a period of 2 years^[35] whilst Schulte^[29] found that they did not discriminate between those with accelerated bone loss and those without. Despite this they may be useful in targeting those patients to treat more aggressively with anti-resorptive agents such as HRT and the bisphosphonates.

Management of patients with IBD and osteoporosis remains problematic although recent guidelines for the management and prevention of osteoporosis in IBD have been published in the United Kingdom^[36]. The guidelines recommend, amongst other measures, that all patients currently taking corticosteroids and with a T score of < -1.5 should be prescribed a bisphosphonate in addition to vitamin D supplementation. This study highlights the potential clinical implications of this strategy, as approximately 20% of our patients would require bisphosphonates if these guidelines were implemented.

We have also established that although there are several significant risk factors for osteoporosis, these combined explain less than 40% of the variability in BMD. A genetic predisposition to osteoporosis complicating other chronic conditions such as rheumatoid arthritis has been described^[37]. This may be of significance in our population and merits further investigation.

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Expressions of sonic hedgehog, patched, smoothened and Gli-1 in human intestinal stromal tumors and their correlation with prognosis

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Abstract

AIM: To investigate the role that the hedgehog (Hh) signaling pathway, which includes sonic hedgehog (Shh), Patched (Ptc), Smoothened (Smo) and Gli-1, plays in human gastrointestinal stromal tumors (GISTs).

METHODS: Surgically resected specimens from patients with GISTs, leiomyomas and schwannomas were examined by immunohistochemical staining for aberrant expression of hedgehog signaling components, Shh, Ptc, Smo and Gli-1, respectively.

RESULTS: In GISTs, 58.1% (18 of 31), 77.4% (24 of 31), 80.6% (25 of 31) and 58.1% (18 of 31) of the specimens stained positive for Shh, Ptc, Smo and Gli-1, respectively. In leiomyomas, 92.3% (12 of 13), 92.3% (12 of 13), 69.2% (9 of 13) and 92.3% (12 of 13) stained positive for Shh, Ptc, Smo and Gli-1, respectively. In schwannomas, 83.3% (5 of 6), 83.3% (5 of 6), 83.3% (5 of 6) and 100% (6 of 6) stained positive for Shh, Ptc, Smo and Gli-1, respectively. Immunohistochemistry revealed that the expressions of Shh and Gli-1 were significantly higher in leiomyomas than in GISTs ($P < 0.05$, respectively). Shh expression strongly correlated with the grade of tumor risk category and with tumor size ($P < 0.05$, respectively). However, the expressions of Ptc and Smo did not correlate with histopathological differentiation.

CONCLUSION: These results suggest that the Hh signaling pathway may play an important role in myogenic differentiation and the malignant potential of human in-

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are rare mesenchymal tumors of the gastrointestinal tract that occur from the esophagus to the anus, including in the omentum, mesentery and retroperitoneum^[1]. Small GISTs are often detected during surgery for other conditions, gastroscopy or routine X-ray^[1,2]. Some GISTs present with bleeding, perforation, pain, obstruction or a combination of these symptoms^[3,4]. These tumors have a wide clinical spectrum from benign, incidentally detected nodules to malignant tumors^[1] categorized into four risk groups: very low, low, intermediate and high^[5]. Traditionally, primary mesenchymal spindle cell tumors of the gastrointestinal (GI) tract have been uniformly classified as smooth muscle tumors (e.g., leiomyomas, cellular leiomyomas, or leiomyosarcomas). Tumors with epithelioid cytologic features have been designated as leiomyoblastomas or epithelioid leiomyosarcomas^[6]. Recently it has been postulated that GISTs originate from Cajal cells in the gastrointestinal tract, which are thought to be pacemaker cells that regulate intestinal motility^[7,8]. Thus, GISTs differ from leiomyomas and schwannomas, which are of mesenchymal cell origin. Further, GISTs are characterized by the frequent expression of the bone marrow leukocytic progenitor cell antigen CD34^[9] and c-kit proto-oncogene^[8]. They also show a remarkable variability in their differentiation, and can be roughly divided into four major categories according to the phenotypic features of the tumors: smooth muscle type, neural type, combined

type and uncommitted type^[10]. Although there are many studies about GISTs, their mechanisms of tumorigenesis, progression and differentiation remain unknown.

The Hedgehog (Hh) gene was initially isolated from *Drosophila* embryonic segments, and it controls patterning of imaginal disc-derived adult structures such as the eye, the appendage and the abdominal cuticle^[11-13]. The mammalian Hh gene, Sonic hedgehog (Shh), is important in the patterning of many tissues and structures such as gastrointestinal epithelium, neurons, smooth muscle tissue, and bone^[14-16]. Shh also plays a role in the development of the endoderm, mesoderm and ectoderm^[17,18]. The response to the Hh signal is controlled by two transmembrane proteins, the tumor-suppressor Patched (Ptc) and the proto-oncogene Smoothened (Smo)^[13]. Smo is a member of the seven transmembrane-receptor family^[11] and its activity is suppressed by the twelve-span transmembrane Ptc. Hh stimulation releases this inhibition, leading to Smo activation of a transcriptional response^[13]. Downstream targets of the pathway in vertebrates include Gli-1, which is associated with development of basal cell carcinomas and medulloblastomas^[19].

There is ample evidence suggesting that the Hh signaling pathway is involved in tumor growth and differentiation. However, there are no studies that examine the expression of Hh pathway components in stromal tumors of the GI tract or the role of the Hh signaling pathway in the etiology of these tumors. Therefore, the purpose of this study is to investigate the expression of Hh pathway signaling proteins in intestinal stromal tumors.

MATERIALS AND METHODS

Tumor classification and selection

A total of 31 GISTs (all cases of stomach), 13 leiomyomas (5 cases of oesophagus, 6 of stomach and 2 of large intestine), and 6 schwannomas (5 cases of stomach and 1 of large intestine) were obtained from patients at Nagasaki University Hospital between 1997 and 2004. The tumor sizes of GISTs were 0.8-8.0 cm in diameter, leiomyomas were 0.1-2.5 cm, and schwannomas were 0.6-4.0 cm. In this study, GISTs were defined and selected as those tumors expressing both c-kit and CD34 surface antigens. Further, we classified smooth muscle actin expressing tumors into smooth muscle (M) type GISTs, S-100 protein expressing tumors into neurogenic (N) type GISTs, both smooth muscle actin and S-100 protein expressing tumors into committed type GISTs, and those expressing only c-kit and CD34 into uncommitted (UN) type GISTs^[10]. And we classified histomorphologically tumors with epithelioid cell form into epithelioid cell (EP) type GISTs, spindle cell form into spindle cell (SP) type GISTs, both epithelioid and spindle cell form tumors into mixed (MIX) type GISTs^[20].

We also categorized GISTs into four groups according to their malignant potential^[5]. The number of mitoses was determined by counting 50 high-power fields ($\times 400$) under Nikon (Tokyo, Japan) E400 microscope. Leiomyomas were defined and selected as tumors expressing smooth muscle actin and not expressing c-kit

and CD34. Schwannomas were defined and selected as tumors expressing S-100 protein and not expressing c-kit and CD34. Tumor identification and classification were determined by two independent pathologists (T. Nakayama and I. Sekine), and cases of questionable diagnosis were omitted from this study.

Immunohistochemical staining

Formalin-fixed paraffin-embedded tissues were cut into 4- μ m sections, deparaffinized in xylene, and rehydrated in PBS. Deparaffinized sections were preincubated with normal bovine serum to prevent non-specific binding and then incubated overnight at 4°C with an optimal dilution (0.1 mg/L) of a primary polyclonal goat antibody against human Shh (N-19), Ptc (C-20), Smo (N-19) and Gli-1 (C-18). Each antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The slides for Shh, Ptc, Smo and Gli-1 were then sequentially incubated with an alkaline phosphatase-conjugated donkey anti-goat immunoglobulin antibody, and the reaction products were visualized using a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride (BCIP/NBT; BRL, Gaithersburg, MD, USA). Primary antibodies preabsorbed with excess antigen peptides or recombinant protein were used as negative controls. Basal cell carcinoma tissue served as the internal positive control for Shh, Ptc, Smo and Gli-1 immunoreactivity. Immunohistochemical analyses were performed independently by two investigators (T. Nakayama and A. Yoshizaki). Shh, Ptc, Smo and Gli-1 expression was classified into two categories depending on the percentage of cells stained: -, 0%-15% positive tumor cells; +, > 15% positive tumor cells.

Statistical analysis

The Stat View II program (Abacus Concepts, Inc., Berkeley, CA, USA) was used for statistical analyses. Analyses comparing the levels of Shh, Ptc, Smo and Gli-1 expression were performed using the Mann-Whitney, Kruskal-Wallis and Spearman's tests. $P < 0.05$ was taken as significant.

RESULTS

The results from the immunohistochemical analysis of Hh pathway components in human intestinal stromal tumors are summarized in Table 1. Shh expression was heterogenous and localized to the cytoplasm of cells in GISTs (Figure 1A). Ptc and Smo were localized to the cytoplasm and cell membrane, and Gli-1 was localized to the cytoplasm and nucleus of GIST tumor cells (Figure 1B-D). Immunohistochemical stainings of Hh pathway components in leiomyomas and schwannomas are shown in Figures 2 and 3, respectively. The four proteins of the Hh pathway that we examined showed patterns of expression similar to that observed in GISTs. While only 58.1% (18 of 31) of the GISTs were positive for Shh, nearly all leiomyomas and schwannomas were positive (92.3%, 12 of 13, $P < 0.05$). The following results were observed for the other proteins: 77.4% (24 of 31), 92.3% (12 of 13), 83.3% (5 of 6) of the GISTs, leiomyomas

Table 1 Aberrant expression of Hedgehog pathway signaling proteins in intestinal stromal tumors *n* (%)

		Shh		Ptc		Smo		Gli-1	
		+	-	+	-	+	-	+	-
GIST	31	18 (58.1)	13 (41.9)	24 (77.4)	7 (22.6)	25 (80.6)	6 (19.4)	18 (58.1)	13 (41.9)
I									
GIST, M	7 (22.6)	4 (57.1)	3 (42.9)	5 (71.4)	2 (28.6)	6 (85.7)	1 (14.3)	4 (57.1)	3 (42.9)
GIST, N	8 (25.8)	5 (62.5)	3 (37.5)	5 (62.5)	3 (37.5)	4 (50.0)	4 (50.0)	3 (37.5)	5 (62.5)
GIST, UN	16 (51.6)	9 (56.3)	7 (43.8)	14 (87.5)	2 (12.5)	15 (93.8)	1 (6.3) ^c	11 (68.8)	5 (31.3)
II									
GIST, EP	5 (16.1)	3 (60.0)	2 (40.0)	4 (80.0)	1 (20.0)	3 (60.0)	2 (40.0)	2 (40.0)	3 (60.0)
GIST, MIX	3 (9.7)	2 (66.7)	1 (33.3)	2 (66.7)	1 (33.3)	3 (100)	0 (0.0)	2 (66.7)	1 (33.3)
GIST, SP	23 (74.2)	13 (56.5)	10 (43.4)	17 (73.9)	6 (26.1)	19 (82.6)	4 (17.4)	14 (60.9)	9 (39.1)
Leiomyoma	13	12 (92.3)	1 (7.7) ^a	12 (92.3)	1 (7.7)	9 (69.2)	4 (30.8)	12 (92.3)	1 (7.7) ^a
Schwannoma	6	5 (83.3)	1 (16.7)	5 (83.3)	1 (16.7)	5 (83.3)	1 (16.7)	6 (100)	0 (0.0)

n (%): Tumor cases followed by percentage (%) of total cases. ^a*P* < 0.05 between Leiomyoma and GIST in Shh or Gli-1; ^c*P* < 0.05 between GIST, UN and GIST, N.

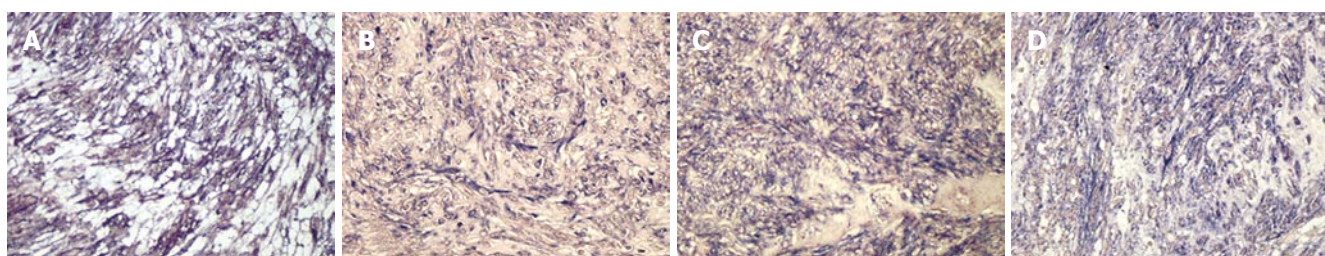


Figure 1 Immunohistochemical staining of Hh signaling components. Alkaline phosphatase reaction products demonstrating Shh (A), Ptc (B), Smo (C) and Gli-1 (D) expression. Shh is expressed in the cytoplasm, Ptc and Smo are expressed in both the cytoplasm and cell membrane, and Gli-1 is expressed in both the cytoplasm and nucleus of GIST cells (x 200).

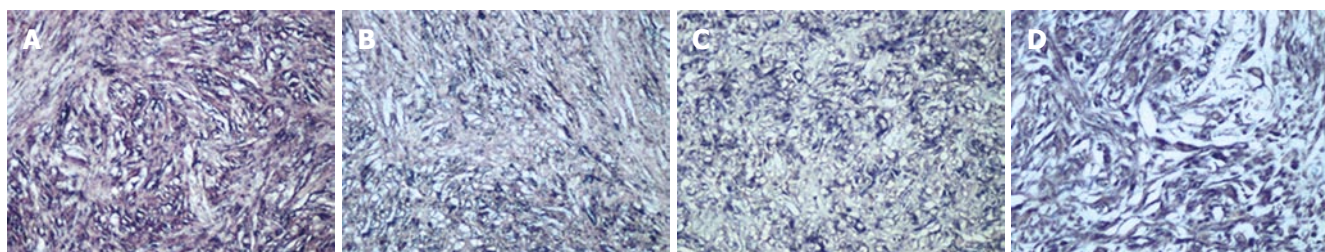


Figure 2 Immunohistochemical staining of human intestinal leiomyomas. Alkaline phosphatase reaction products demonstrating Shh (A), Ptc (B), Smo (C) and Gli-1 (D) expression (x 200).

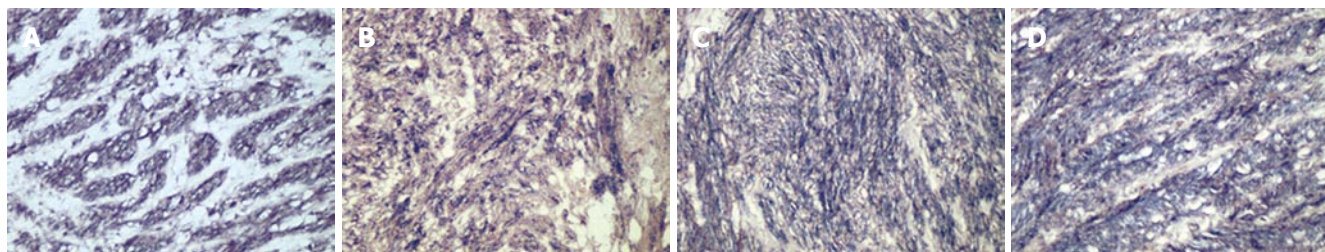


Figure 3 Immunohistochemical staining of human intestinal schwannomas. Alkaline phosphatase reaction products demonstrating Shh (A), Ptc (B), Smo (C) and Gli-1 (D) expression (x 200).

and schwannomas were positive for Ptc, respectively; 80.6% (25 of 31), 69.2% (9 of 13), 83.3% (5 of 6) of the GISTs, leiomyomas and schwannomas were positive for Smo, respectively; and 58.1% (18 of 31), 92.3% (12 of 13), 100.0% (6 of 6) of the GISTs, leiomyomas and

schwannomas were positive for Gli-1, respectively. Though schwannomas expressed high levels of each protein examined, they had no statistical correlation with GISTs or leiomyomas.

Immunohistochemical analyses of Hh pathway com-

Table 2 Expression of Hedgehog pathway components in various categories of intestinal stromal tumors *n* (%)

	<i>n</i>	Shh		Ptc		Smo		Gli-1	
		+	-	+	-	+	-	+	-
GIST	31	18 (58.1)	13 (41.9)	24 (77.4)	7 (22.6)	25 (80.6)	6 (19.4)	18 (58.1)	13 (41.9)
Risk categories		<i>P</i> < 0.05		NS		NS		NS	
High	4	1 (25.0)	3 (75.0)	3 (75.0)	1 (25.0)	3 (75.0)	1 (25.0)	3 (75.0)	1 (25.0)
Intermediate	6	3 (50.0)	3 (50.0)	5 (83.3)	1 (16.7)	4 (66.7)	2 (33.3)	2 (33.3)	4 (66.7)
Low	16	9 (56.3)	7 (43.8)	11 (68.8)	5 (31.3)	13 (81.3)	3 (18.8)	9 (56.3)	7 (43.8)
Very low	5	5 (100)	0 (0.0)	5 (100)	0 (0.0)	5 (100)	0 (0.0)	4 (80.0)	1 (20.0)
Tumor size (cm in diameter)		<i>P</i> < 0.05		NS		NS		NS	
<i>n</i> ≤ 2	8	7 (87.5)	1 (12.5)	7 (87.5)	1 (12.5)	8 (100)	0 (0.0)	6 (75.0)	2 (25.0)
2 < <i>n</i> ≤ 5	18	10 (55.6)	8 (44.4)	14 (77.8)	4 (22.2)	13 (72.2)	5 (27.8)	8 (44.4)	10 (55.6)
5 < <i>n</i>	5	1 (20.0)	4 (80.0)	3 (60.0)	2 (40.0)	4 (80.0)	1 (20.0)	4 (80.0)	1 (20.0)
Mitosis counts ¹		NS		NS		NS		NS	
0-5	23	13 (56.5)	10 (43.5)	17 (73.9)	6 (26.1)	18 (78.3)	5 (21.7)	14 (60.9)	9 (39.1)
6-10	3	2 (66.7)	1 (33.3)	3 (100)	0 (0.0)	3 (100)	0 (0.0)	2 (66.7)	1 (33.3)
11-28	5	3 (60.0)	2 (40.0)	4 (80.0)	1 (20.0)	4 (80.0)	1 (20.0)	2 (40.0)	3 (60.0)

n (%): Tumor cases followed by percentage (%) of total cases; NS: Not significant; ¹Numbers per 50 areas in high-power field (× 400).

ponents in GISTs classified by cellular differentiation are shown in Table 1. In this study there was no case where a GIST was composed of combined types of cellular differentiation. The following results were observed: Shh was detected in 56.3% (9 of 16), 57.1% (4 of 7) and 62.5% (5 of 8) of UN, M and N type of GISTs; Ptc was detected in 87.5% (14 of 16), 71.4% (5 of 7) and 62.5% (5 of 8), respectively; Smo was detected in 93.8% (15 of 16), 85.7% (6 of 7) and 50.0% (4 of 8), respectively; and Gli-1 was detected in 68.8% (11 of 16), 57.1% (4 of 7) and 37.5% (3 of 8), respectively. The expression of Smo was significantly lower in UN type than in N type of GISTs (*P* < 0.05). And immunohistochemical analyses of Hh pathway components in GISTs classified histomorphologically by cellular form are shown in Table 1, also. In all GISTs, 16.1% (5 of 31) of EP cell type, 9.7% (3 of 31) of MIX cell type and 74.2% (23 of 31) of SP cell type were included, respectively. Each Hh pathway component was detected variably in different cellular type of GISTs. However, there was no correlation between the expression of Hh pathway components and cellular subtype of GISTs.

The results from immunohistochemical analysis of Hh pathway components with regard to the malignant potential of GISTs are summarized in Table 2. There were no correlations between mitosis counts and the expression levels of Hh pathway components. In contrast, the results suggested that lower levels of Shh expression correlated with lower risk GIST categories (*P* < 0.05) and smaller tumor sizes (*P* < 0.05).

DISCUSSION

Recent studies have shown that the Hh pathway plays important roles in cell differentiation, tissue patterning and embryonic development^[16,19,21]. However, the role of the Hh pathway in human intestinal stromal tumors is still unclear. We investigated the expression of Shh, Ptc, Smo and Gli-1 in three types of intestinal stromal tumors (GISTs, leiomyomas and schwannomas) using immunohistochemical techniques. Our data demonstrated

that Shh and Gli-1 were expressed at higher levels in leiomyomas than in GISTs. It suggested that Shh and Gli-1 expressions were correlated with myogenic differentiation. However, in the subclassification of GISTs, myogenic differentiation did not show expression of Shh or Gli-1. Moreover, the consequence of low expression of Smo in neuronal GISTs is not clear yet. Thus, future studies will address the role of the Hh pathway in the differentiation of intestinal stromal tumors.

Abrogation of the Hh pathway can also lead to tumorigenesis. In this pathway, Gli-1, which is involved in controlling cell proliferation and angiogenesis, is a key target of oncogenic action^[3,13,22]. Loss of function Ptc mutations and gain of function Smo mutations are mechanisms of tumorigenesis in many types of tumors such as basal cell carcinomas, medulloblastomas, astrocytomas, small cell lung carcinomas and pancreatic cancers^[19,23-25].

In this study, the expression of Shh correlated with low risk categories and small tumor sizes. It suggests that expression of Shh reduces the risk of malignant GISTs. Normally, Shh releases Smo from Ptc suppression to induce Gli-1 expression and activation^[11,13,19]. Then by a negative feedback mechanism, Gli-1 suppresses the expression of Shh, which results in decrease of Gli-1. However, our data did not show a concomitant decrease in Gli-1 expression in tumors that expressed low levels of Shh. In fact, we observed high Gli-1 levels in larger tumors of high risk categories when Shh expression was low. We hypothesize that Gli-1 may be up-regulated by pathways other than the Hh pathway, or mutation of an Hh pathway component could disrupt the feedback mechanism in high risk GISTs. Thus, future studies will examine Hh pathway components in high risk GIST tumors. In conclusion, our study suggests that the Hh pathway may play important roles in myogenic differentiation and the malignant potential of human intestinal stromal tumors.

In recent studies, mutations affecting c-kit that cause constitutive tyrosine kinase activation have been shown to be important for the pathogenesis of GIST^[26,27]. Joensuu

et al.^[28] reported a patient in whom Imatinib (STI-571, Gleevec), a tyrosine kinase inhibitor, was effective against a GIST. And Imatinib has been proven to be remarkably efficacious in heavily pretreated GISTs patients with advanced disease in phase III clinical trials^[29]. The expression of the Hh pathway is upregulated by the activation of tyrosine kinase through the epidermal growth factor pathway^[30], and may be upregulated by the c-kit/tyrosine kinase pathway.

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CLINICAL RESEARCH

Treatment of hepatitis C virus genotype 4 with peginterferon alfa-2a: Impact of bilharziasis and fibrosis stage

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bilharzial and non-bilharzial patients in both groups. In terms of safety and tolerability, neutropenia was the predominant side effect; both drugs were comparable.

CONCLUSION: PegIFN- α 2a combined with ribavirin results in improvement in sustained response in HCV genotype 4, irrespective of history of bilharzial infestation.

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Key words: Hepatitis C virus; Genotype 4; Pegasys; Bilharziasis

Derbala MF, Al Kaabi SR, El Dweik NZ, Pasic F, Butt MT, Yakoob R, Al-Marri A, Amer AM, Morad N, Bener A. Treatment of hepatitis C virus genotype 4 with peginterferon alfa-2a: Impact of bilharziasis and fibrosis stage. *World J Gastroenterol* 2006; 12(35): 5692-5698

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Abstract

AIM: To evaluate pegylated interferon alpha2a (PegIFN- α 2a) in Egyptian patients with HCV genotype 4, and the impact of pretreatment viral load, co-existent bilharziasis and histological liver changes on response rate.

METHODS: A total of 73 naïve patients (61 with history of bilharziasis) with compensated chronic HCV genotype 4 were enrolled into: group A (38 patients) who received 180 mg PegIFN-alpha2a subcutaneously once weekly for a year and group B (35 patients) received IFN alpha-2a 3 MU 3 times weekly. Ribavirin was added to each regimen at a dose of 1200 mg. Patients were followed for 72 wk and sustained response was assessed.

RESULTS: Significant improvement in both end of treatment response (ETR) ($P < 0.002$) and sustained response (SR) ($P < 0.05$) was noted with pegylated interferon, where ETR was achieved in 29 (76.3%) and 14 patients (40%) in both groups respectively, and 25 patients in group A (65.8%) and 9 (25.7%) in group B could retain negative viraemia by the end of follow up period. Sustained virological response (SVR) showed a significant negative correlation with age and positive correlation with pretreatment inflammation in patients receiving PegIFN. Viral clearance after 3 mo of therapy was associated with high incidence of ETR and SR ($P < 0.001$), but without significant difference between both forms of interferon. Significant improvement in response was achieved in patients with high grade fibrosis (grade 3 and 4) with PegIFN- α 2a, where SR was seen in 5 out of 13 patients in group A, but none in group B. There was no significant difference in response between

INTRODUCTION

Hepatitis C is comparable to a 'viral time bomb'. The WHO estimates that about 200 million people, 3% of the world's population, are infected with hepatitis C virus (HCV) and 3 to 4 million persons are newly infected each year. The striking genetic heterogeneity of RNA genome of HCV is well recognized. Six major genotypes and over 50 subtypes and minor variants referred to as "quasispecies" are described^[1]. HCV genotype differences seem to be of considerable clinical significance because they affect the responses to antiviral therapy^[2]. HCV genotype 4 appears to be prevalent in the Middle East and Central Africa, where almost 13% of HCV carriers around the world live in the Eastern Mediterranean region. Prevalence rates of HCV genotype 4 ranges from 60% in Saudi Arabia to 90% in Egypt where it has been reported to be frequently associated with cirrhosis and a poor response to interferon (IFN)^[3,4].

Concurrent HCV-genotype 4 infection and schistosomiasis result in a much more severe liver disease than that seen with either disease alone. Luckily, the activity of HCV infection seems to be partially suppressed in such patients^[5]. The effect of such co-infection on hepatic fibrosis and in turn on response to treatment in HCV patients is however, conflicting. While Helal *et al* in 1989^[6] and Shiha *et al* in 2002^[7] reported a lack of enhancement

of hepatic pathology in the schistosomal patients, Hassan *et al* in 2002^[8] suggested that schistosomiasis is an important risk factor involved in enhancement of nitric oxide levels and virus replication, which in turn may aggravate liver cell injury and hence the development of cirrhosis.

It has been reported that treatment with conventional IFN is less effective in patients with genotypes 1 and 4 than in patients with genotypes 2 and 3^[9]. The high rate of HCV turnover coupled with the short half-life of the drug, limits the efficacy of conventional IFN therapy^[10]. Pegylated IFN- α 2a [Peg-IFN- α 2a (40 kDa); Pegasys, Hoffmann-La Roche] is produced by attachment of a 40 kDa branched polyethylene glycol moiety to IFN- α 2a by a stable amide bond. It is characterized by prolonged absorption half-life, restricted volume distribution, and decreased clearance compared to standard interferon, which thus increase its therapeutic efficacy with less frequent doses^[11]. Recent clinical trials have shown that the response to pegylated interferon α 2a plus ribavirin (RBV) therapy for chronic HCV infection is superior to that achieved with standard interferon α 2a plus ribavirin therapy or peginterferon- α 2a alone with a rapid decline in viral load in the first 12 wk, for all HCV genotypes^[12]. Hematological adverse effects in the form of anaemia, neutropenia and thrombocytopenia are the primary laboratory abnormalities experienced during IFN plus RBV combination therapy and may necessitate dose modification and thus potentially impact outcome. This anemia is attributed to both ribavirin dose-dependent hemolysis and direct suppressive effect of interferon on erythropoiesis^[13]. Hematopoietic growth factors may be useful in the management of these side effects.

The purpose of this prospective analysis is to compare the effectiveness and safety of Pegasys (40 kDa) IFN- α 2a once weekly with IFN- α 2a, in compensated HCV genotype 4, in combination with ribavirin. The effect of pretreatment viral load, histological liver changes and schistosomiasis co-infection on response to treatment is also assessed.

MATERIALS AND METHODS

Patients

Adult patients with chronic active hepatitis C as evidenced by positive serological test for HCV-Ab using enzyme linked immunosorbent assay (ELISA) (Ortho Diagnostics, Neckargmun, Germany), detectable serum HCV-RNA by RT-PCR (Amplicor Molecular System, Hoffmann-La Roche, Basel, Switzerland), elevated serum alanine transaminases (ALT) activity more than twice the normal value and histopathological criteria of chronic active hepatitis. Liver histology was classified according to Scheuer score system from 0-4 for both grades (necroinflammation) and stage (degree of fibrosis). Hepatocellular carcinoma was excluded by testing of α -fetoprotein and by ultrasound scanning. None of the female patients was pregnant as evidenced by negative serum pregnancy test. Breast feeders were excluded. All patients had normal serum direct and indirect bilirubin, albumin and creatinine. All patients were genotype 4

detected by the Inno LiPA HCV II assay (Innogenetics Inc., GA, USA). Patients were excluded if co-infected with HBV, HIV. Hemochromatosis, Wilson disease or other causes for chronic liver disease were also ruled out. Other exclusion criteria included neutrophil count $< 1.5 \times 10^9$ /L, platelet count $< 90 \times 10^9$ /L or haemoglobin (Hb) < 100 g/L for female and < 110 g/L for male, positive auto-antibodies including antinuclear antibody (ANA), antimitochondrial antibody (AMA), anti-smooth muscle autoantibody (ASMA), patients with a history of severe psychiatric disease, seizure disorder, organ transplantation, or severe cardiac or pulmonary disease. All patients had normal thyroid function prior to the study and all were either non-diabetics or with controlled blood glucose level with hemoglobin A_{1c} $< 8.5\%$. Patients were excluded if they had clinically significant retinal abnormalities, clinical gout, were a substance abuser (alcohol or I.V. drugs) or showed any medical condition requiring systemic steroids.

Safety assessment

Patients were reviewed in the Hepatology Outpatient Clinic weekly during the first month and monthly thereafter along the course of therapy to check for safety, and then followed for at least 6 mo after discontinuation of treatment to assess for sustained response. Epoetin beta (Recormon®. Roche) at a dose of 4000 U/weekly for 2 wk was given when Hb level decrease > 30 mg/L or $> 25\%$ from baseline levels. Also Filgrastim (Neupogen®. Amgen. Inc. F. Hoffmann-La Roche Ltd. Basel) 5 μ g/kg was given once or twice weekly if neutrophils $< 0.7 \times 10^3/\mu$ L, while drug was discontinued completely for any patient showing Hb level < 85 g/L, neutrophils $< 0.5 \times 10^9$ /L, platelet $< 50 \times 10^9$ /L, abnormal thyroid function tests, creatinine > 177 μ mol/L or ALT/AST double baseline levels. Patients requiring modification of more than 4 doses were excluded.

Efficacy assessment

The primary efficacy end point was sustained response (SR), defined as undetectable HCV RNA and normal ALT level at the end of follow up (24 wk after discontinuation of treatment). The relapse rate was calculated as percentage of patients with an end-of-treatment response in whom HCV RNA was detectable at wk 72. End of treatment response (ETR) was defined as normalization of ALT and loss of detectable serum HCV RNA at the end of treatment.

Study design

This randomized, controlled clinical trial was conducted from February 2002 to November 2004. The study consisted of a screening phase, which began 2 mo prior to the first dose of the drug under evaluation. Examination established eligibility of patients according to inclusion/exclusion criteria. After a written informed consent was obtained in accordance with the Helsinki Declaration of 1979, 80 patients were randomly assigned at a 1:1 ratio to receive either, subcutaneously, once weekly 180 μ g of peginterferon- α 2a (Pegasys, Hoffmann-LaRoche) or IFN α 2a (Roferon®, Hoffmann-LaRoche) 3 MU 3 times. Ribavirin 1200 mg at a daily oral dose was added to both

Table 1 Demographic data of the hepatitis C patients

Variable	Group A (PEG-IFN + RBV) n = 38	Group B (IFN + RBV) n = 35	P
Age (yr) (mean ± SD)	45.5 ± 6.1	45.4 ± 5.8	NS ¹
Gender			
Male	31 (81.6)	33 (94.3)	
Female	7 (18.4)	2 (5.7)	
Body mass (mean ± SD)			
Before treatment	81.9 ± 12.0	73.6 ± 7.7	0.001
After Treatment	78.9 ± 12.5	71.1 ± 7.4	0.002
Weight reduction	2.9 ± 4.3	2.6 ± 2.7	NS ¹
Inflammation stage			
0-1	7 (18.4)	8 (22.9)	NS ¹
2-3	31 (81.6)	27 (77.1)	
Grade of fibrosis			
Mild (0-2)	25 (65.8)	23 (65.7)	NS ¹
Severe (3-4)	13 (34.2)	12 (34.3)	
Bilharzial co-infection No.	31 (81.6)	30 (5.7)	

¹Not significant.

regimens. Throughout the study, patients were monitored for vital signs, weight, adverse events, medication compliance, thyroid function, haematologic parameters, blood chemistry and serum HCV- RNA levels.

Statistical analysis

The data were coded, and processed on an IBM-PC compatible computer using Statistical Packages for Social Sciences (SPSS). Data were expressed as mean and standard deviation (SD) unless otherwise stated. Student-t-test was used to ascertain the significance of difference between mean values of two continuous variables and Mann-Whitney test was used for non-parametric distribution. Chi-Square analysis was performed to test for differences in proportions of categorical variables between 2 or more groups. In 2 × 2 tables, the Fisher exact test (two-tailed) was used instead of Chi-Square, in particular, when sample size was small. One-way analysis of variance (ANOVA) and non-parametric Kruskal Wallis one-way analysis of variance (ANOVA) was employed for comparison of several group means and to determine the presence of significant differences between group means. The Pearson's correlation coefficient was used to evaluate the strength association between two variables. The level $P < 0.05$ was considered as the cut-off value for significance. Multivariate logistic regression analysis was performed.

RESULTS

Seventy-three patients out of 80 completed the study and follow up periods, and were classified into 2 groups: 38 patients received pegylated IFN and ribavirin (group A) and 35 received non-pegylated IFN and ribavirin (group B). Seven patients (2 in group A and 5 in group B) could not continue the study because of severe side effects or intolerance to treatment. Thyroid dysfunction in one patient in each group, intolerance of the drug's side effect in another one in group A and in 2 patients from

Table 2 Comparison of response of hepatitis C in both groups

Variable	Group A (PEG-IFN + RBV) n = 38	Group B (IFN + RBV) n = 35	P
After 48 wk			
Responders	29 (76.3)	14 (40.0)	< 0.002
Non-responders	9 (23.7)	21 (60.0)	
After 72 wk			
Among responders			
Sustained response	25 (86.2)	9 (64.3)	0.124
Relapser	4 (13.8)	5 (35.7)	

group B, in addition to increase of transaminases and thrombocytopenia in 2 patients with cirrhosis in group B were the causes of drug discontinuation. Male gender was predominant in both groups, 31 and 33 respectively. Baseline demographic data and disease characteristics were similar in both groups (Table 1). Thirty-one patients in group A and 30 in group B had a history of bilharziasis treated with either tarter emetic (44 patients) or praziquentel (17 patients). Among these, one had histological pattern of bilharzial granuloma in liver tissue, but none had active bilharziasis prior to treatment.

Patients who received pegylated IFN (29, 76.3%) showed a significant ETR in comparison to those receiving non-peg-IFN (14, 40%) ($P < 0.002$). A significant ($P < 0.05$) improvement in SR was noticed with Peg-IFN, where 25 patients in group A (65.8% of total number of patients who completed the study) and 9 (25.7 %) in group B could retain negative viraemia at the end of follow up period (Table 2). A significant negative correlation between age and sustained response was noted in both groups ($P = 0.015$) without a significant difference between both drugs. There was no correlation between gender, pre-treatment, viraemia or body weight and response rate (Table 3). There was a significant positive correlation between pre-treatment ALT and ETR in patients receiving Peg-IFN but not in patients receiving IFN. Also, a significant positive correlation ($P < 0.05$) was found between stage of hepatic inflammation and response rate in patients treated with peg-IFN, but not with IFN (Table 3). Intent to treat (ITT) analysis showed significant improvement in both of ETR and SR with peg-IFN therapy, where ETR was 72.5% and 35%, respectively, while SR was 62.5% and 22.5% ($P < 0.002$).

Regarding viraemia, there was no significant difference between responders and non-responders in both groups and within the same group. Viral clearance after 12 wk of therapy was associated with high incidence of ETR and SR ($P < 0.001$), but also, without significant difference between both groups. By studying the relation between histopathological activity and response, treatment with peg-IFN showed a significant improvement in response and sustained response in patients with severe fibrosis (grade 3 and 4). Only one patient out of 11 with severe fibrosis showed ETR with conventional IFN therapy and unfortunately relapsed after discontinuation of treatment, while 8 patients out of 13 showed ETR with peg-IFN therapy and 5 of them could retain negative viremia at

Table 3 Comparison between responders and non-responders in both hepatitis C groups (mean \pm SD)

Variable	Group A (PEG-IFN + RBV) <i>n</i> = 38			Group B (IFN + RBV) <i>n</i> = 35		
	R ¹	NR ²	SR ³	R ¹	NR ²	SR ³
Age (yr)	44.7 \pm 5.4	47.8 \pm 7.9	44.2 \pm 5.8	45.23 \pm 5.2	45.6 \pm 6.2	42.9 \pm 4.6
Body mass (kg)	82.6 \pm 10.2	79.7 \pm 17.0	82.7 \pm 9.8	74.6 \pm 9.5	73.1 \pm 6.6	77.3 \pm 9.6
ALT (μ kat/L)						
Before treatment	2.559 \pm 1.564	1.524 \pm 0.082	2.757 \pm 1.607	1.494 \pm 0.432	1.810 \pm 0.570	1.524 \pm 0.515
After 3 mo	1.065 \pm 0.980	1.095 \pm 0.767	1.084 \pm 1.039	0.405 \pm 0.112	1.135 \pm 0.741	0.487 \pm 0.263
Baseline HCV RNA (MU/L)	473 934 \pm 373 542	496 256 \pm 667 356	472 444 \pm 402 109	312 786 \pm 185 583	361 857 \pm 339 942	346 667 \pm 207 364

¹Responders; ²Non responder; ³Sustained response.

Table 4 Comparison of response in both hepatitis C groups according to histopathological changes *n* (%)

Variable	<i>n</i>	Responders	Non responders	Among responders	
				Sustained response	Relapse
Severe Fibrosis					
A ¹	13	8 (61.5)	5 (38.5)	5 (62.5)	3 (37.5)
B ²	11	1 (9.1)	10 (90.9)	0 (0.0)	1 (100.0)
Mild fibrosis					
A ¹	25	21 (84.0)	4 (15.0)	20 (95.2)	1 (4.8)
B ²	24	13 (54.2)	11 (45.8)	9 (69.2)	4 (30.8)
Severe inflammation					
A ¹	31	22 (71.0)	9 (29.0)	18 (81.8)	4 (18.2)
B ²	27	7 (25.9)	20 (74.1)	4 (57.1)	3 (42.9)
Mild inflammation					
A ¹	7	7 (100.0)	0 (0.0)	7 (100.0)	0 (0.0)
B ²	8	7 (87.5)	1 (12.5)	5 (71.4)	2 (28.6)

¹Group A = PEG-IFN+RBV; ²Group B = IFN + RBV.

72 wk (Table 4). There was no significant difference in response in bilharzial and non-bilharzial patients in both groups, where SR was achieved in 27 patients co-infected with bilharziasis (60.7%) and in 7 cases of HCV alone (58.3%) (Table 5).

With respect to safety and tolerability, peg-IFN was comparable to conventional IFN. Weight reduction was similar in both groups, where the mean reduction was 2.9 \pm 4.3 kg and 2.6 \pm 2.7 kg respectively. After flu-like picture, hematological side effects represented the commonest encountered problem (Table 6). Although anemia was seen in 71.1% and 65.7% in both groups respectively, only 39.5% and 37.1% in both groups developed Hb drop more than 30 mg/L or > 30% of baseline level and required growth stimulating factors (These figures were related only to those patients who completed the study). Two patients from group B with cirrhotic changes were withdrawn because of thrombocytopenia. The proportions of patients withdrawn from treatment because of laboratory abnormalities or other adverse effects were similar in both groups and no new or unexpected adverse effects specific to peginterferon were presented. On multivariate logistic regression analysis, no significant predictive values were noted.

DISCUSSION

Hepatitis genotype is now recognized as the most

important baseline characteristic determining treatment regimen and the most useful predictor of response^[14]. Slow viral dynamics, particularly second-phase decay^[15] and limited effectiveness of IFN in blocking the virion have been implicated in poor response to IFN therapy in hepatitis C genotype 4 patients^[16], which genotype has been described as a difficult-to-treat one. Unfortunately, this is the predominant genotype in the Middle East where large numbers of affected individuals are reported. A great improvement in response has recently been noted in all genotypes after the introduction of pegylated forms of IFN, whether as monotherapy or combined with ribavirin. Data presented in this study further reinforce the superior efficacy of PegIFN/Ribavirin combination therapy in terms of ETR and SVR in HCV genotype 4 infection. This is in accordance with earlier reports (Thakeb *et al* 2004^[17]; Shobokshi *et al* 2004^[18]; and Diago *et al* 2004^[19]). The lower SR noted compared to that reported by Diago *et al*^[19] for western genotype 4 cases (65.8% *vs* 79%) may reflect a difference in sensitivity of genotype 4 subtypes to PegIFN- α 2a. The improved response to PegIFN- α 2a in genotype 4 compared to conventional IFN and PegIFN- α 2a previously reported by us^[20,21] can be attributed, at least in part, to a high and persistent trough serum level of PegIFN- α 2a in the first 4 wk of treatment which led to rapid viral eradication^[22,23], or the recently reported third phase decay with PegIFN- α 2a in the first 1-4 wk of therapy^[24]. Other causes also include

Table 5 Comparison between responders and non-responders in both hepatitis C groups

Variable	Responders <i>n</i> = 43	Non Responders <i>n</i> = 30	<i>P</i>
Gender			
Male	38 (88.4)	26 (86.7)	NS ¹
Female	5 (11.6)	4 (13.3)	
Body mass (kg, Mean ± SD)			
Before treatment	80.1 ± 10.6	75.1 ± 10.9	NS ¹
After treatment	76.9 ± 10.6	72.9 ± 11.5	NS ¹
ALT			
Pre treatment	141.1 ± 88.9	101.2 ± 41.0	NS ¹
12 wk	56.2 ± 55.0	67.1 ± 44.0	NS ¹
48 wk	38.1 ± 22.7	65.0 ± 30.6	NS ¹
PCR (No. of viruses in BL; mean ± SD)			
Pre treatment	416328 ± 341106	337321 ± 482826	0.036
Liver fibrosis			
Grade 1	17 (39.5)	3 (10.3)	0.009
Grade 2	17 (39.5)	11 (37.9)	
Grade 3	8 (18.6)	8 (27.6)	
Grade 4	1 (2.4)	7 (24.1)	
HCV vs HCV and Bilharziasis			
Co-infected	37 (84.1)	24 (82.8)	NS ¹
Non Bilharzial	7 (15.9)	5 (17.2)	
Drug used			
Pegasus	29 (67.4)	9 (30.0)	0.002
Non-pegylated IFN	14 (32.6)	21 (70.0)	

¹Not significant.

the improved patients' adherence to treatment and the use of erythropoietin and G-CSF, thus overcoming anaemia and neutropenia that have commonly led to halting therapy.

The significant correlation noted in the present study between viral clearance at wk 12 and SVR, regardless of the type of IFN used, confirms a consistent relationship between the rapidity of HCV-RNA suppression and the likelihood of achieving SR^[25,26]. Conversely, patients showing positive PCR at wk 12, all failed to achieve SR regardless of ETR seen in 2 of them. This suggests that a positive PCR at wk 12 in genotype 4 cases might be considered as a strong negative predictor of response.

In terms of pretreatment predictors, only patient's age showed a negative correlation with response rate. This is probably not related to age *per se*, but rather to the age of infection, or in other words, the duration of infection, since older patients are known to develop a higher rate of liver fibrosis^[27]. The presence of cirrhosis has been shown to be independently associated with decreased SVR in HCV infected patients^[28]. In this respect we could demonstrate a superior SR with PegIFN- α 2a over non-pegylated IFN (25.7% *vs* 0%) in both advanced fibrosis and compensated cirrhosis genotype 4 patients. Similar results were reported by Heathcote *et al* in 2000^[29] and Marcellin *et al* in 2004^[30].

In agreement with Tsubota *et al*^[31] and contrary to Picciotto *et al*^[32], we could not find any correlation between pretreatment viral load and SR, which implies that HCV-

Table 6 Comparison of side effects in both hepatitis C groups

Variable	Group A	Group B	<i>P</i>
Flu-like symptoms	40 (100.0)	40 (100.0)	
Discontinuation of drug	0 (0)	0 (0)	NS ¹
Thyroid dysfunction	2	3	NS ¹
Discontinuation of drug	1	2	
Psychological upset and drug intolerance	1	1	NS ¹
Discontinuation of drug	1	1	
Hematological			
Anemia (30% from baseline)	13 (26.0)	12 (24.0)	NS ¹
Neutropenia ($< 0.9 \times 10^9/L$)	21 (42.0)	19 (38.0)	NS ¹
Thrombocytopenia ($< 50 \times 10^9/L$)	None	2 (4)	NS ¹

¹Not significant.

RNA levels *per se* are less influential compared to the major impact of genotype that generally determines the rate of SR.

HCV patients co-infected with schistosomiasis exhibited a unique clinical, virological and histological pattern manifested by an increased incidence of viral persistence with high HCV-RNA titers and accelerated fibrosis. This may be attributed to the fact that patients with schistosomiasis have a down regulated immune response to HCV in the form of reduced IFN γ , IL-4 and IL-10 secreted by HCV-specific T cells^[33]. In spite of this, we did not find any significant difference in response to either IFN forms in cases of combined infections. This might be explained by the recent observation of El Rafei and colleagues^[34] that *Schistosoma mansoni* by targeting a specific subset of memory CD8 cells, reduces the late differentiated memory T cell population in HCV co-infected individuals. This implies that patients infected with the genotype 4 can still mount HCV-specific T cell responses, despite the prevalence of concomitant schistosomiasis.

As for safety and tolerability, both IFN forms were comparable. As in previous reports, anemia and thrombocytopenia were the commonest hematological adverse events of the combination therapy^[35]. Nevertheless, none of our patients experienced bleeding tendency or uncontrolled infection throughout the study period. The use of Epoietin α and G-CSF helped improve patients' adherence to treatment, and minimize dose reduction and discontinuation of treatment in the first 12 wk. Adherence to therapy is increasingly recognized as a key determinant in the outcome of antiviral therapy in chronic hepatitis^[36] and although erythropoietin stimulates both erythropoiesis and thrombopoiesis^[37], the latter effect was not demonstrated in our patients and 4 had to discontinue treatment because of thrombocytopenia.

We can conclude that concomitant HCV-genotype 4 and bilharzial infections do not seem to affect the improved responses achieved with pegylated interferon α 2a plus ribavirin combination therapy. Also, in spite of the improved response in advanced fibrosis and compensated cirrhosis, advanced histopathological changes, coupled with positive viremia after 12 wk of

therapy, still remain the most important negative predictive factor for response in genotype 4 patients. A non-stop and extensive work is still needed to win the battle against HCV. Each new pharmacological modification carries with it more hope for better control of this complicated disease and tells that the difficult to treat genotype 4 will eventually be conquered.

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Endoscopic mucosal resection of large hyperplastic polyps in 3 patients with Barrett's esophagus

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Abstract

AIM: To report the endoscopic treatment of large hyperplastic polyps of the esophagus and esophagogastric junction (EGJ) associated with Barrett's esophagus (BE) with low-grade dysplasia (LGD), by endoscopic mucosal resection (EMR).

METHODS: Cap fitted EMR (EMR-C) was performed in 3 patients with hyperplastic-inflammatory polyps (HIPs) and BE.

RESULTS: The polyps were successfully removed in the 3 patients. In two patients, with short segment BE (SSBE) (≤ 3 cm), the metaplastic tissue was completely excised. A 2 cm circumferential EMR was performed in one patient with a polyp involving the whole EGJ. A simultaneous EMR-C of a BE-associated polypoid dysplastic lesion measuring 1 cm x 10 cm, was also carried out. In the two patients, histologic assessment detected LGD in BE. No complications occurred. Complete neosquamous re-epithelialization occurred in the two patients with SSBE. An esophageal recurrence occurred in the remaining one and was successfully retreated by EMR.

CONCLUSION: EMR-C appears to be a safe and effective method for treating benign esophageal mucosal lesions, allowing also the complete removal of SSBE.

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Key words: Hyperplastic polyps; Endoscopic mucosal resection; Barrett's esophagus

INTRODUCTION

Benign esophageal tumors are rare, representing less than 1% of all esophageal tumors^[1]. They are usually asymptomatic and often discovered incidentally^[2]. Many classifications have been proposed for benign esophageal tumors: by histological cell types, which are divided into epithelial and non-epithelial tumors, or by the location in the esophageal wall, categorized as intramural and extramural tumors^[3,4].

Epithelial polypoid lesions of the esophagus and esophagogastric junction (EGJ) are uncommon^[5-8]. Among them, hyperplastic-inflammatory polyps (HIPs) often occur in combination with gastroesophageal reflux disease (GERD)^[9-13]. Endoscopic mucosal resection (EMR) is a promising therapeutic option for the removal of superficial carcinomas throughout the gastrointestinal tract. This technique permits resection of the mucosa and submucosa, exposing the muscularis propria^[14]. The authors report the outcome of EMR in three patients with large hyperplastic polyps of the EGJ associated with Barrett's esophagus (BE).

MATERIALS AND METHODS

Endoscopy

Upper gastrointestinal endoscopy (UGIE) was performed with standard forward-viewing videoendoscope (GIF-Q145, Olympus Optical Co. Ltd., Tokyo, Japan).

Before EMR, endoscopic ultrasonography (EUS) (GF-UMQ 130, 7.5-20 MHz, Olympus Optical Co. Ltd., Tokyo, Japan) was performed to assess lesion depth and lymph node status.

Endoscopic resection technique

EMR was performed with the "cap" technique (EMR-C). A 13 mm diameter transparent plastic cap (MH-594, Olympus Co. Ltd., Tokyo, Japan) was preloaded on the tip of a standard diagnostic forward-viewing endoscope



Figure 1 A sessile villous-like polyp extending for the whole length of BE.

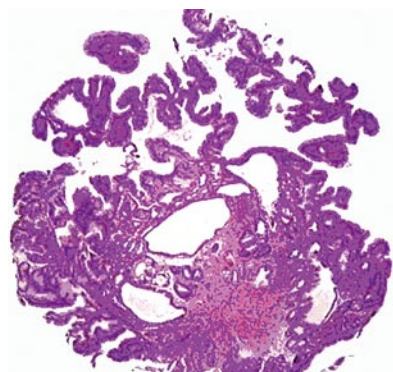


Figure 2 Hyperplastic polyp from the gastroesophageal junction was comprised of cardiac type mucosa with foveolar hyperplasia and cystic dilatation of gastric pits (HE x 100).

(GIF-Q145, Olympus Optical Co. Ltd., Tokyo, Japan). Inside the distal end of the cap is a gutter that positions the opened monofilament polypectomy snare (SD-221U-25, Olympus Co. Ltd., Tokyo, Japan). After submucosal injection of diluted epinephrine (1:200 000), with methylene blue, the cap was pressed against the mucosa and suction applied. The polypectomy snare was closed around the tissue and resection performed by endocut mode only, using the ERBE-ICC 200 cautery device (ERBE Elektromedizin Gmgh, Tubingen, Germany).

After each resection the endoscope was withdrawn to collect the tissue specimen.

Deep sedation with propofol was used to perform the endoscopic procedures.

RESULTS

All resected fragments were fixed in 40 g/L neutral formaldehyde, embedded in paraffin and serially sectioned. The sections were stained with hematoxylin and eosin; in addition all specimens were stained with periodic acid-Schiff/Alcian blue stain at pH 2.5 for evaluation of intestinal metaplasia (IM).

Immunohistochemical staining for p53 was performed in all cases (clone DO-7, BioGenex, San Ramon, CA; final dilution 1:20 000).

Case 1

A 72-year old man complained of gastroesophageal reflux symptoms since the age of 25, which became worse in the last five years, occurring also during the night. He was taking over-the-counter drugs (ranitidine) for his reflux symptoms, with poor control. He never complained of dysphagia. The past medical history of the patient was unremarkable. Endoscopy showed a 3 cm hiatal hernia, and a circumferential BE 10 cm long. A sessile villous-like polyp, 10 mm wide, extending for the whole length of BE, was observed (Figure 1). The colour of the lesion was similar to BE, and at the EGJ became wider, involving the entire circumference of the junction for a length of 15 mm.

EUS demonstrated a lesion confined to the mucosa. No pathologic lymph nodes were detected. Multiple biopsies from BE showed incomplete IM with low-grade epithelial dysplasia (LGD), rare pancreatic pits and positivity for p53; biopsies from the EGJ polyp showed proliferation

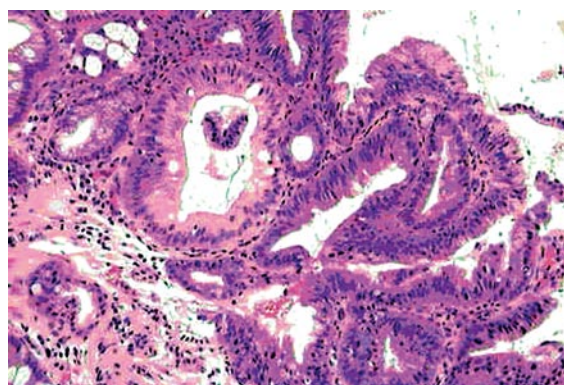


Figure 3 Sessile lesion arising in the Barrett's esophagus: the adenomatous villous-type polyp is composed of low grade dysplastic epithelium (HE x 250).

of hyperplastic cardiac-type epithelium with some cystic dilatation of gastric pits, rare goblet cells, rare parietal cells (Figure 2) and some groups of cells with LGD. Additional features included oedema and inflammation in the lamina propria.

Biopsies from the sessile lesion in BE showed fragments of adenomatous villous-type with incomplete IM, epithelial LGD (Figure 3) and over expression of p53.

The patient was hospitalised and EMR-C performed. Both lesions (the long esophageal one, 1 cm × 10 cm, and its 2 cm of circumferential expansion at the EGJ), were completely removed in one endoscopic session. No intraprocedural or delayed complications occurred. On histology, the EGJ lesion was a hyperplastic polyp, and the lesion arising in BE was a villous adenomatous polyp with LGD (also named BE-associated polypoid dysplastic lesion).

Intravenous omeprazole was administered during the following 24 h. Then 40 mg of rabeprazole were given. The patient was kept fasting for 24 h. Then a liquid diet was started, and solid foods were reintroduced five days later. He was dismissed after four days on long term rabeprazole 40 mg.

UGIE was not repeated until 18 mo later, as the patient refused earlier endoscopic examinations. He was asymptomatic, and still taking 40 mg of rabeprazole. Endoscopy showed a 10 cm long area of neosquamous re-epithelialization where EMR had been performed. Three areas of mucosal abnormality (average size: 12 mm) were detected in the lower esophagus (Figure 4), and EMR was repeated, with resection of larger areas of metaplastic epithelium to favour neosquamous re-epithelialization. No

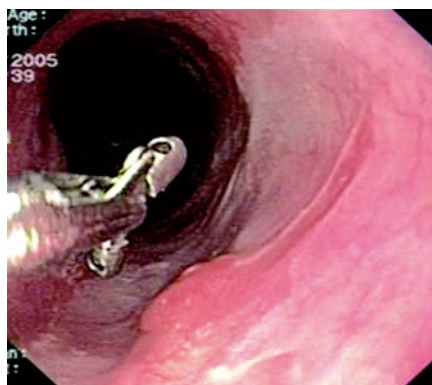


Figure 4 Areas of neosquamous re-epithelialization coexisting with tiny mucosal abnormalities.

complications occurred and the patient left the hospital three days later. Histology showed both hyperplastic cardiac-type and cystic hyperplastic gastric fundic polyps. The resected associated BE showed incomplete IM without dysplasia.

A further endoscopy performed 3 mo later, revealed large areas of re-epithelialization and no mucosal abnormalities in the residual BE. Multiple biopsies were taken from the residual BE, that showed IM without dysplasia.

Case 2

A 66-year old woman underwent endoscopy for recurrent mild epigastric pain during the previous three months, without gastroesophageal reflux symptoms. The past medical history was unremarkable. Endoscopy showed a 3 cm hiatal hernia, a short segment BE (SSBE-20 mm), and a 25 mm sessile polyp at the EGJ. Multiple biopsies showed hyperplastic squamous epithelium. EUS showed a lesion confined to the mucosa, without lymph node involvement. Biopsies of the BE showed incomplete IM with LGD, and p53 negativity.

The patient was hospitalised and EMR-C performed. The polyp and BE were both excised. No early or delayed complications occurred. The patient left the hospital three days later, and rabeprazole 40 mg was advised. The first repeat endoscopy was scheduled after 3 mo, when histology revealed proliferative hyperplastic squamous epithelium and chronic inflammatory cells in the lamina propria. Endoscopy 3 and 6 mo later showed complete neosquamous re-epithelialization of the lower esophagus, without stricture.

Case 3

A 72-year old man underwent endoscopy for recurrent epigastric pain occurring over the previous five years, without reflux symptoms. Endoscopy showed a 30 mm tongue of metaplastic epithelium in the distal esophagus, a 3 cm hiatal hernia, and a 30 mm sessile polyp at the EGJ. Biopsies from the BE and polyp showed incomplete IM and squamous epithelial hyperplasia. EUS showed a lesion confined to the mucosa. No pathologic lymph nodes were detected.

Both polyp and BE were completely removed by EMR-C. No complications occurred and the patient left

the hospital three days later. He was advised to assume 40 mg of rabeprazole indefinitely. Histology confirmed that the polyp consisted of hyperplastic squamous epithelium and inflammatory infiltrate. The resected BE showed incomplete IM without dysplasia. Immunohistochemical stain for p53 was negative. Endoscopy 3 and 6 mo later showed complete neosquamous re-epithelialization of the lower esophagus, with minimal scarring.

DISCUSSION

We were able to remove large polyps of the esophagus and EGJ by EMR in 3 patients with BE, without complications. In the two patients with a SSBE, the metaplastic tissue was completely removed, allowing complete neosquamous re-epithelialization.

At histology, inflammatory polyps are characterized by hyperplastic epithelium with variable amounts of inflamed stroma^[5]. Occasionally, esophageal polyps containing stroma and granulation tissue, without an epithelial component, have been reported^[5-7]. Other types of polyps are the inflammatory pseudotumors, also known as inflammatory polyps with pseudomalignant erosion. They usually occur in the distal esophagus, arise from the mucosal layer and contain inflamed granulation tissue with bizarre stromal cells. Pseudomalignant erosion has been found in 14.3% of inflammatory polyps of the EGJ^[15]. Inflammatory esophagogastric polyps are thought to be a complication of gastroesophageal reflux^[6,16]. They have also been reported in patients with hiatal hernia (88%) and/or reflux esophagitis (91%)^[6,17]. The association of hyperplastic polyps and BE is infrequent, and to our knowledge, only Abraham *et al*^[5] has reported it previously. In fact, adenoma is more frequently associated with BE. It has been suggested that the use of the term “adenoma” in BE may be misleading, because it carries a “benign” connotation^[18]. The appropriate term for these lesions should be “BE-associated polypoid dysplastic lesion”, because they share similar clinical, pathological and molecular features as those of flat dysplasia^[19].

In a series of 27 patients with a total of 30 hyperplastic esophageal and EGJ polyps, 80% of them were composed of cardiac-type mucosa, 17% of squamous mucosa, and 3% contained both cardiac and squamous mucosa. IM was present in only two polyps (7%)^[5]. The location of HIPs was the EGJ region (67%), distal esophagus (30%) and mid-esophagus (3%), in accordance with other data reported in the literature^[16,17].

Only four cases of hyperplastic polyps in the cervical esophagus have been reported^[20-23], all arising from ectopic gastric mucosa^[24,25].

Less often, polyps are located at the end of a prominent inflamed gastric fold very close to the squamocolumnar junction, also known as “the sentinel fold”, seen in reflux esophagitis^[26].

Histologically similar polyps have been reported in von Recklinghausen's^[27], and Crohn's disease^[28,29], protracted vomiting^[5,30], and infectious esophagitis (Candida, Cytomegalovirus, Herpes simplex virus)^[5]. Other esophageal injuries, due to drugs (K-dur, alendronate sodium and ibuprofen)^[5], sclerotherapy for esophageal

varices^[12], polypectomy, and photodynamic therapy^[5], can result in hyperplastic polyp occurrence. Their pathogenesis is similar to that of gastric and colonic hyperplastic polyps that frequently seem to occur in response to mucosal injury^[31,32]. However, there are cases in which the etiology cannot be defined^[5,33]. Malignant transformation has been occasionally reported in gastric and colonic hyperplastic polyps^[32,34], but not in those arising in the esophagus or EGJ.

In the study of Abraham *et al*^[5], 4 of 27 patients with esophageal hyperplastic polyps also had BE. IM within cardiac-type mucosa was present in two polyps (6.7%): one contained goblet cells, and the other one was surrounded by Barrett's mucosa. Only the latter polyp (3%) showed LGD. Three out of 4 patients with BE had previous, concomitant, or subsequent development of dysplasia in the non-polypoid esophagus. Furthermore, the frequency of dysplasia within esophageal hyperplastic polyps is low and it has been reported only in those cases associated with dysplastic Barrett's esophagus^[5].

In our patients, histological evaluation showed hyperplastic epithelium and inflamed stroma in the polyps located at EGJ. Histological assessment of the 10 cm long polyp arising in BE showed a "BE-associated polypoid dysplastic lesion". The treatment of HIPs of the esophagus and EGJ has changed over the years. In the past open surgical resection was considered standard care in the management of all significant esophageal lesions^[2,33]. Transabdominal Nissen fundoplication operation to prevent reflux has also been used in cases where the correlation between HIPs and GERD was documented^[11].

More recently thoracoscopic approaches have been performed for mucosal or submucosal lesions > 30 mm^[2]. Endoscopic polypectomy for HIPs is the current treatment for lesions ≤ 30 mm^[15,16,27]. Oguma *et al*^[21] reported the successful removal of a pedunculated hyperplastic polyp (size: 9.0 mm × 4.5 mm × 4.0 mm) arising in the ectopic gastric mucosa in the cervical esophagus^[23]. Two of our patients had LGD in the BE. In patients with BE, cancer can develop through several steps encompassing LGD, high-grade dysplasia (HGD) and invasive adenocarcinoma^[35]. However, adenocarcinomas have also been detected in patients whose previous biopsies revealed only low-grade or even no dysplasia^[36-39]. The information on the natural history of LGD is limited. Moreover, the diagnosis of LGD is highly subjective and associated with interobserver variability^[40]. In three articles, the reported rate of progression from LGD to HGD or adenocarcinoma was 10%^[41], 12%^[42], and 28%^[37]. In these studies the agreement among pathologists in diagnosing LGD was associated with an increased risk of progression to HGD or cancer.

At present, patients with HGD are advised to undergo surgical treatment due to the reported frequency of undetected synchronous cancers^[43,44]. However, surgery carries substantial morbidity and mortality^[45]. EMR could become a therapeutic alternative to esophagectomy in selected patients^[46-50].

Some authors suggest EMR in patients with BE and LGD to improve the histologic accuracy^[46,51]. However, complications can occur during this procedure. Bleeding

and perforation have been reported in a median 10% of patients and less than 1% of cases, respectively^[50]. Esophageal stenosis is a late complication, and may occur in 0%-30% of cases^[50]. Larger EMR resections may increase the risk of stenosis. In a study encompassing 137 patients, stenosis occurred only when EMR involved more than two-thirds of the esophageal circumference^[52]. However, in one report of circumferential EMR, only two of 12 patients developed stenosis^[53]. Overall, complications seem fewer for EMR than for surgical resection^[54].

We were able to remove the large sessile lesions with EMR-C, in one session, without complications. In two of our patients, with non-circumferential SSBE, the metaplastic epithelium was completely removed. The patient with a 10 cm long BE showed a large neosquamous re-epithelialization, involving half of the BE surface. In the same patient the 20 mm circumferential EMR at the EGJ level did not cause any stricture.

In conclusion, our experience indicates that esophageal and EGJ hyperplastic polyps could represent an exuberant epithelial regeneration following mucosal injury, particularly GERD. The presence of BE in all of them seems to confirm the hypothesis of GERD in causing inflammatory reaction. The malignant transformation of these polyps is rare, but possible^[5]. Also, the presence of dysplasia within a segment of BE is patchy, and random biopsies may fail to detect it^[55].

We performed EMR to remove the large hyperplastic polyps and the surrounding areas of BE, as EMR provides greater diagnostic precision than endoscopic biopsy. In the study of Conio *et al*^[50] reclassification of the histology after EMR occurred in 26% in a series of 39 patients with BE. Other authors have reported reclassification of the histologic diagnosis after EMR in 0% to 75% of cases^[53,56]. We can conclude that EMR is minimally invasive and a low risk method in treating large benign esophageal mucosal lesions.

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Assessment of oxidative stress in chronic pancreatitis patients

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Abstract

AIM: To assess the levels of antioxidant capacity and oxidative damage in blood of chronic pancreatitis (CP) patients in comparison with those in healthy control subjects, by using several different analytical techniques.

METHODS: Thirty-five CP patients and 35 healthy control subjects were investigated prospectively with respect to plasma levels of thiols, ferric reducing ability of plasma (FRAP, i.e. antioxidant capacity), levels of protein carbonyls and thiobarbituric acid reactive substances (TBARS). Additionally, we evaluated the production of reactive oxygen species (ROS) in whole blood.

RESULTS: The antioxidative thiols including cysteine, cysteinylglycine and glutathione were significantly lower in CP patients. In addition, the non-enzymatic antioxidant capacity was significantly lower in CP patients, which correlated with the amount of oxidative protein (protein carbonyls) and the extent of lipid damage (TBARS), both were significantly higher in CP patients. The ROS production in whole blood after stimulation with phorbol 12-myristate 13-acetate, demonstrated a strong tendency to produce more ROS in CP patients.

CONCLUSION: Oxidative stress may contribute to the pathogenesis of chronic pancreatitis by decreasing antioxidant capacity and increasing oxidative damage in CP patients may be a rationale for intervention with antioxidant therapy.

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Key words: Chronic pancreatitis; Oxidative stress; Thiols; Ferric reducing ability of plasma; Protein carbonyls; Thiobarbituric acid reactive substances; Reactive oxygen species

INTRODUCTION

Chronic pancreatitis (CP) is a progressive irreversible inflammatory disease that eventually leads to an impaired exocrine and/or endocrine function of the pancreas^[1-4]. Although most cases have been attributed to alcohol abuse, the underlying causes of CP appear to be multi-faceted, including environmental as well as genetic factors. Chronic pancreatitis shares risk factors with pancreatic cancer such as smoking and alcohol abuse, but itself is also a risk factor for pancreatic adenocarcinoma^[5]. A genetic predisposition to pancreatitis is supported by the identification of sequence alterations in the genes encoding cationic trypsinogen (PRSS1), the cystic fibrosis transmembrane conductance regulator (CFTR), and the serine protease inhibitor, Kazal type 1 (SPINK1) in patients with hereditary or idiopathic chronic pancreatitis^[1,6-8]. Additionally, an increased frequency of SPINK1 mutations been reported in patients with alcohol-related chronic pancreatitis^[3,9]. So far we have not completely understood the pathogenesis of CP^[10]. Different hypotheses have been proposed, including the contribution of oxidative stress of endogenous origin or chemical stress by environmental or lifestyle-related xenobiotics^[11-15]. There is growing recognition that an imbalance between reactive oxygen species (ROS) producing and ROS scavenging processes leads to the damage of pancreatic acinar cells, initiating auto-digestion of the entire pancreas. This insight is suggested by data from experimental and clinical studies^[16-19]. Oxidative stress may be important in the pathogenesis of ethanol-induced pancreatic injury, although radiation, exposure to cigarette smoke, medication or trauma may stimulate the generation of free radicals, which subsequently may result in damage of lipids, proteins or nucleic acids. Activation of (enzymatic) antioxidative defence has been described in pancreatic disease independent of its origin^[20]. Glutathione and cysteine are important mediators in the defence against oxidative stress and both molecules play a key role in the maintenance of cellular thiol redox status. Therefore, in the present study the concentrations of glutathione, cysteine and other thiols were measured in blood plasma of patients with CP

and healthy control subjects. In addition, we also measured the non-enzymatic antioxidant capacity by applying the ferric reducing ability of plasma (FRAP) assay in patients with CP and healthy controls. Further assessment of the level of oxidative stress was performed by measuring the concentrations of protein carbonyls in plasma in order to determine the amount of oxidative protein damage. As an indicator of lipid peroxidation we established the concentrations of thiobarbituric acid-reactive substances (TBARS). Finally, we investigated the generation of ROS by chemiluminescence in whole blood of both patients and controls.

MATERIALS AND METHODS

Subjects

The study was approved by the local medical ethical review committee and all subjects gave their written informed consent. This study was conducted at the Department of Gastroenterology of the University Medical Centre Nijmegen, the Netherlands and all subjects studied were Caucasians of Dutch extraction. A total of 35 consecutive CP patients were recruited between January 2004 and June 2004 at the out-patient clinic of the department. In 29 patients an alcohol-related etiology was indicated (ACP), the remaining 6 CP patients had a family history of CP (HCP). The clinical diagnosis of CP was based on one or more of the following criteria: presence of typical complaints (recurrent upper abdominal pain, radiating to the back, relieved by leaning forward or sitting upright and increased after eating), suggestive radiological findings such as pancreatic calcifications or pseudocysts, and pathological findings (pancreatic ductal irregularities and dilatations) revealed by endoscopic retrograde pancreatography or magnetic resonance imaging of the pancreas before and after stimulation with secretin. ACP was diagnosed in patients who consumed more than 60 g (females) or 80 g (males) of ethanol per day for more than two years before they were diagnosed, during their treatment they all gave up drinking alcohol. HCP was diagnosed based on the presence of two first-degree relatives or three or more second-degree relatives in two or more generations, suffering from recurrent acute pancreatitis or chronic pancreatitis for which there was no precipitating factor. For comparison, we collected a control group consisting of 35 healthy subjects. We recruited our healthy controls by advertisement in a local paper and did not apply any monetary incentive for the controls to participate.

Analysis of thiols

Samples of blood were taken by venapuncture into EDTA tubes. Whole blood was centrifuged at $1500 \times g$ for 10 min within 1 h after collection and plasma was stored at -30°C until analysis. Concentrations of the thiols including cysteine, homocysteine, cysteinylglycine and glutathione (the sum of reduced-, oxidised- and protein-bound thiols) in plasma were quantified using high performance liquid chromatography (HPLC) with fluorescent detection, essentially as described by Fortin *et al*^[21] and modified by Raijmakers *et al*^[22]. Thiol levels were calculated using four-point calibration curves for each thiol, which were run in

parallel with the samples, and values were expressed in $\mu\text{mol/L}$.

Analysis of FRAP

The antioxidant capacity in blood plasma was measured using the ferric reducing ability of plasma (FRAP) assay, according to the method of Benzie and Strain^[23]. The reduction of ferric to ferrous ion at low pH formed a coloured ferrous-tripyridyltriazine complex. Absorbance changes were linear over a wide concentration range with antioxidant mixtures, including plasma. FRAP values were obtained using a seven-point calibration curve of known amounts of Fe^{2+} and expressed in $\text{mmol Fe}^{2+}/\text{L}$.

Analysis of protein carbonyls

The amount of oxidative protein damage, as a marker for oxidative stress, was determined using an enzyme linked immunosorbent assay (ELISA) for estimation of protein carbonyls in body fluids, as essentially described by Buss *et al*^[24] and adapted by Zusterzeel *et al*^[25]. Samples were incubated with dinitrophenylhydrazine and then adsorbed to wells of an ELISA plate before probing with a commercial antibody raised against protein-conjugated dinitrophenylhydrazine. The binding of biotin-conjugated primary antibody was then quantified after incubation with streptavidin-biotinylated horseradish peroxidase and staining with o-phenylenediamine. Calibration took place using oxidised and fully reduced albumin, and carbonyl levels were expressed in $\mu\text{mol/g protein}$.

Analysis of TBARS

Thiobarbituric acid-reactive substances (TBARS), mainly malondialdehyde (MDA) in plasma were evaluated by recording the fluorescence spectrum between 500 and 600 nm on a Shimadzu RFF-5000 spectrofluorometer, of the thiobarbituric acid-malonaldehyde complex, as described by Conti *et al*^[26]. Levels of TBARS were expressed in $\mu\text{mol MDA}/\text{L}$.

Analysis of ROS

ROS production in whole blood was evaluated using luminol-enhanced chemiluminescence, as measured in an automated LB96V Microlumat Plus Luminometer (EG & G Berthold, Belgium). Briefly, the signal-amplifying molecule luminol reacts with oxygen species (mainly superoxide anion) generated by neutrophils in whole blood, to produce an excited state intermediate that emits light as it returns to its ground state. ROS production was determined in the absence of a cellular stimulator, as well as in the presence of either a receptor-dependent (serum-treated zymosan, STZ) or a receptor-independent stimulus (phorbol 12-myristate 13-acetate, PMA). Freshly obtained heparinized blood was 1:100 diluted in HBSS containing 1 mmol/L calcium. Two hundred μL of this diluted blood was added to each well of a 96-well plate. In addition, reaction mixtures contained 0.45 g/L bovine serum albumin (BSA), 0.83 mmol/L luminol and either 1 g/L STZ, 0.4 mg/L PMA or no stimulating agents. As an internal positive control for the luminescence process, samples of 1 g/L ammonium persulphate (APS) in phosphate-buffered

solution (PBS) were run simultaneously. Chemiluminescence was monitored every 60 s for 1 h. EDTA blood, taken together with the heparinized blood samples, was tested for leukocyte counts and differentiation, in order to adjust the chemiluminescence produced during one hour ('area under the curve') in relative light units (RLU) per cell for neutrophil counts. All measurements were performed in quadruplicate and corrected for background values (absence of a stimulus). Opsonized zymosan particles were prepared by incubation of STZ with pooled human serum for 30 min at 37°C, as previously described^[27]. These particles were then washed twice in PBS and finally suspended at 12.5 g/L in PBS.

Statistical analysis

Data were analysed using SPSS version 12.0. Differences in the baseline characteristics of patients and controls were estimated with Fisher's exact test and Student's *t*-test. The Mann-Whitney U-test was used to estimate differences in biochemical parameters between the patient and control population in a non-parametrical manner. Differences were considered significant if $P < 0.05$. Finally, we examined the correlation between the non-enzymatic antioxidant capacity with the amount of oxidative protein and lipid damage in CP patients, using Spearman rank correlation test.

RESULTS

The characteristics of patients with CP and healthy controls are denoted in Table 1. The mean age of the CP patients was 51 years (range 25 to 74 years) and was not significantly different from that of the healthy controls (45 years; range 27 to 68 years). There was no significant difference in the distribution of gender between CP patients and healthy control subjects. Smoking habits between CP patients and healthy controls were not different; 66% of the patients and 63% of the control subjects smoked or stopped smoking within the last 5 years.

The oxidative stress was measured in CP patients and healthy controls. The plasma concentrations of cysteine (Cys), homocysteine (Hcys), cysteinylglycine (CGS) and glutathione (GSH), the plasma antioxidant capacity (FRAP) as well as the plasma levels of protein carbonyls and TBARS and chemoluminescence in whole blood are depicted in Table 2.

The plasma concentrations of antioxidative thiols including cysteine, cysteinylglycine and glutathione were significantly lower in the CP patients than in the controls ($P = 0.021$, $P = 0.003$ and $P = 0.048$, respectively). The plasma levels of homocysteine were similar in both groups. The antioxidant capacity as measured by the FRAP assay was also significantly lower in the CP patients than in the healthy control subjects ($P < 0.001$). The levels of both carbonyls and TBARS were significantly higher in the CP patients than in the healthy controls ($P < 0.001$). The chemiluminescence of diluted whole blood of CP patients and controls was not different, although there was a strong trend towards an increased ROS production after stimulation with PMA ($P = 0.058$). As expected, spontaneous generation of ROS in the absence of a stimulus was less

Table 1 Main characteristics of patients with chronic pancreatitis and healthy controls

Characteristic	CP patients	Controls
<i>n</i>	35	35
Gender		
Male/Female	17/18	18/17
Mean age (yr) (range)	51(25-74)	45 (27- 68)
Smoking		
Yes/No	23/12	22/13

Table 2 Thiol plasma concentrations, FRAP antioxidant capacity, protein carbonyl plasma levels, TBARS plasma levels and ROS production in whole blood of patients with CP and controls mean (range)

Measure for oxidative stress	CP patients	Controls
Cys ¹ (μmol/L)	225 (124-314) ^a	249 (212-328)
Hcys ² (μmol/L)	13.6 (5.0-38.2)	12.7 (0.2-27.8)
CGS ³ (μmol/L)	34.8 (23.5-124) ^a	39.3 (25.2-56.7)
GSH ⁴ (μmol/L)	7.5 (2.4-18.5) ^a	8.9 (3.5-16.1)
FRAP ⁵ (mmol Fe ²⁺ /L)	0.75 (0.31-1.73) ^a	0.99 (0.69-1.57)
Carbonyls (nmol/mg protein)	0.32 (0.02-1.47) ^a	0.04 (0.01-0.07)
TBARS ⁶ (μmol/L)	4.98 (0.23-27.79) ^a	0.35 (0.04-0.68)
ROS ⁷ production (RLU/10 ⁴ cells)		
PMA ⁸ -stimulated	40 (1-83)	33 (1-87)
STZ ⁹ -stimulated	124 (11-266)	111 (9-2130)

¹Cysteine; ²Homocysteine; ³Cysteinylglycine; ⁴Glutathione; ⁵Ferric reducing ability of plasma; ⁶Thiobarbituric acid-reactive substances; ⁷Reactive oxygen species; ⁸Phorbol 12-myristate 13-acetate; ⁹Serum treated zymosan. ^a $P < 0.05$ vs control.

than 10% of the amount of ROS measured in response to the stimuli of PMA and STZ. The leukocyte counts and differentiation within the ranges were considered normal at our hospital. The values obtained with either assay were not different in patients with ACP and HCP.

In addition, the correlation between the non-enzymatic antioxidant capacity with the amount of oxidative protein and lipid damage in CP patients was examined and a negative correlation was found between the non-enzymatic antioxidant capacity and the amount of oxidative protein damage ($r_s = -0.44$, $P = 0.013$), as well as between the non-enzymatic antioxidant capacity and the amount of lipid damage ($r_s = -0.39$, $P = 0.004$). Finally, we found a positive correlation between oxidative protein and lipid damage ($r_s = 0.67$, $P = 0.001$).

DISCUSSION

Alcohol abuse is regarded as a major risk factor for the development of CP. However, the exact mechanism behind the effect of alcohol remains unknown. Some evidence obtained by animal studies, suggests that metabolism of ethanol catalysed by cytochrome P4502E1 (CYP2E1) may contribute to oxidative stress in the pancreas during chronic alcohol consumption^[19]. Trauma, exposure to radiation, cigarette smoke, medication or other toxins generating free

radicals also may increase the amount of oxidants. In the present study, we assessed the antioxidant capacity and levels of oxidative damage in CP patients as compared with healthy controls by means of several analytical techniques that are known to measure various components that together constitute oxidative stress. The major observations were that plasma concentrations of some thiols, having antioxidant properties, were significantly lower in CP patients. Likewise the non-enzymatic antioxidant capacity as measured by the FRAP assay, was significantly lower in CP patients than in healthy control subjects. This inferior antioxidant capacity in CP patients parallels significantly increased amounts of oxidative protein and lipid damage, whereas the generation of ROS in whole blood did not show a statistically significant difference between CP patients and healthy control subjects, with a similar age and gender distribution and smoking habits. Our results clearly indicate that oxidative stress is present in patients with CP and that this eventually might contribute to the initiation and maintenance of inflammation in CP patients, as has been previously suggested^[17,19,28-31]. Thiols such as cysteine and glutathione play an essential role as antioxidants, and are involved in protein synthesis, redox sensitive transduction signalling, cell growth and proliferation, xenobiotic metabolism and immune regulation^[32]. Glutathione is conjugated to many xenobiotics and essential for the optimal functioning of numerous enzymes and hence crucial for cell viability. Decreased levels of glutathione in plasma have been reported, but paradoxically also increased glutathione levels may be found as a result of outshoot after enhanced synthesis due to oxidative stress or conjugation of toxic compounds, as has been shown in different disorders^[33-35]. We found significantly lower plasma concentrations of cysteine, cysteinylglycine and glutathione in CP patients as compared to healthy controls, whereas the control values measured here can be considered normal for the Dutch population^[36]. We found no elevated concentrations of homocysteine in CP patients, however homocysteine does not always act as an antioxidant, moreover elevated plasma concentrations of homocysteine are positively associated with an increased risk of cerebral, coronary or peripheral vascular diseases^[36]. In parallel with the lower plasma concentrations of the antioxidative thiols, the FRAP assay also demonstrated a significantly lower antioxidant capacity in CP patients. Since the FRAP assay does not measure sulfhydryl (SH)-containing antioxidants such as the thiols glutathione and cysteine, this indicates that other non-thiol related antioxidants are decreased in CP patients also. In patients with acute pancreatitis very low ascorbate concentrations in plasma have been described before^[37,38].

Protein carbonyl derivatives are generated by direct oxidative attack of proteins or by indirect lipid peroxidation products and therefore represent a good biomarker for general oxidative stress^[39,40]. The lower FRAP levels in CP patients are accompanied with high protein carbonyl concentrations, indicating that increased oxidative damage occurs as a result of the lower protective capacity as measured by FRAP. Former studies have demonstrated

elevated plasma protein carbonyls in experimental animal models^[41,42] and humans with acute pancreatitis^[43]. Unless properly scavenged, ROS may lead to lipid peroxidation, which represents an important manifestation of oxidative stress. Lipid peroxidation is initiated when free radicals interact with polyunsaturated fatty acids. For instance, in cell membranes this may result in a chain reaction forming lipid hydroperoxides. Analysis of TBARS in plasma is a widely used method for the estimation of lipid peroxidation. In accordance with the elevated levels of protein carbonyls and lower antioxidant capacity, we found significantly increased plasma concentrations of TBARS in CP patients. The production of ROS, as measured in whole blood by chemiluminescence assay, was not significantly higher in CP patients than in healthy controls, although there was a strong trend to generate higher amounts of ROS after stimulation with PMA. We used luminol-enhanced chemiluminescence assay in 100-fold diluted whole blood to study ROS generation of peripheral blood cells in a non-disturbed system. As expected, ROS generation in the absence of a stimulus in CP patients was low, and not significantly different from the values in the healthy control group. Chemiluminescence measurements did not demonstrate a significantly increased ROS generation in CP patients, while the other analytical techniques applied here showed increased oxidative stress and damage in CP patients as compared to healthy control subjects, demonstrating that the oxidative damage in CP patients is caused by other reactive (oxygen) species rather than by leucocytes. Since most of the CP patients included in this study were alcoholics, the cause of oxidative damage might be mainly of exogenous origin. However, a contribution of oxidative stress of endogenous origin might also be possible, since we detected a strong tendency to produce ROS in CP patients after stimulation with PMA. This PMA-induced respiratory burst is receptor-independent and not absolutely dependent on priming^[44], whereas priming does moderately influence the STZ-induced respiratory burst^[45]. We measured the ROS production in whole blood and it is known that during isolation of neutrophils, these cells often become primed^[46]. The clinical importance of oxidative stress in human pancreatic disease was first suggested by Braganza *et al*^[47], and subsequently supported by data from other groups, showing increased levels of lipid hydroperoxides in pancreatic juice^[14] and increased spontaneous production of ROS by neutrophils^[27,48]. The assessment of oxidative stress in CP patients corroborates the hypothesis that oxidative stress leads to damage of pancreatic acinar cells, initiating auto-digestion of the entire pancreas as has been shown in the present study.

In summary, significantly higher levels of products of oxidative damage (protein carbonyls and TBARS), correlating with decreased levels of cysteine, cysteinylglycine, glutathione and non-enzymatic antioxidant capacity (FRAP) can be found in CP patients. Oxidative stress, defined as the imbalance between prooxidant and antioxidant capacity, is higher in CP patients, which may justify further studies on intervention with antioxidant therapies for this serious disease.

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Chronic Epstein-Barr virus-related hepatitis in immunocompetent patients

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Abstract

AIM: To investigate reactivated Epstein-Barr virus (EBV) infection as a cause for chronic hepatitis.

METHODS: Patients with occasionally established elevated serum aminotransferases were studied. HIV, HBV and HCV-infections were excluded as well as any other immunosuppressive factors, metabolic or toxic disorders. EBV viral capsid antigen (VCA) IgG and IgM, EA-R and EA-D IgG and Epstein-Barr nuclear antigen (EBNA) were measured using IFA kits. Immunophenotyping of whole blood was performed by multicolor flow cytometry. CD8⁺ T cell responses to EBV and PHA were determined according to the intracellular expression of IFN- γ .

RESULTS: The mean alanine aminotransferase (ALT) and gamma glutamyl transpeptidase (GGTP) values exceeded twice the upper normal limit, AST/ALT ratio < 1. Serology tests showed reactivated EBV infection in all patients. Absolute number and percentages of T, B and NK cells were within the reference ranges. Fine subset analysis, in comparison to EBV⁺ healthy carriers, revealed a significant decrease of naive T cells ($P < 0.001$), accompanied by increased percentage of CD45RA⁺ ($P < 0.0001$), and terminally differentiated CD28⁺CD27⁺ CD8⁺ T cells ($P < 0.01$). Moderately elevated numbers of CD38 molecules on CD8⁺ T cells ($P < 0.05$) proposed a low viral burden. A significantly increased percentage of CD8⁺ T cells expressing IFN- γ in response to EBV and PHA stimulation was registered in patients, as compared to controls ($P < 0.05$). Liver biopsy specimens from 5 patients revealed nonspecific features of low-grade hepatitis.

CONCLUSION: Chronic hepatitis might be a manifestation of chronic EBV infection in the lack of detectable immune deficiency; the expansion of CD28⁺CD27⁺ and increase of functional EBV-specific CD8⁺ T cells being the only surrogate markers of viral activity.

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Key words: Chronic hepatitis; Epstein-Barr; Epstein-Barr virus-specific CD8⁺ T cell

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INTRODUCTION

Epstein-Barr virus (EBV) infects more than 90% of humans by the time of adulthood and persists as a life-long latency, suggesting sophisticated mechanisms for effective immune evasion. Primary EBV infection is usually asymptomatic or results in infectious mononucleosis, which resolves spontaneously after the emergence of EBV-specific immunity. Similar to other herpes viruses, EBV persists in the infected immunocompetent organism, without causing apparent disease. CD8⁺ T cell-mediated responses control the virus both during primary infection and the following carrier state^[1,2].

So far, studies on the interaction between the immune system and EBV have focused mostly on congenital or acquired immune deficiency states, which can lead to viral reactivation. Rare cases of fulminant chronic EBV infection have been described and termed severe chronic active EBV infection (SCAEBV or CAEBV)^[3,4]. Criteria of CAEBV include severe infection lasting more than 6 mo, histologic evidence of major organ involvement, abnormal EBV antibody titers, and increased quantities of EBV in affected tissues in apparently immunocompetent hosts^[5]. However, congenital or acquired defects of cell-mediated immune mechanisms in CAEBV patients are very probable, associated with signalling defects, reduced T-cell or NK cell activity, or reduced perforin levels^[6].

While hepatitis is a common feature of primary EBV infection^[7,8], the role of EBV in chronic liver disease is less obvious. Bertolini *et al* showed typical histopathological lesions of chronic hepatitis without involvement of other organs after inoculation of normal human bone marrow-derived B cell, carrying an endogenous EBV line *in vivo* in nu/nu mice^[9]. Single cases of persistent hepatitis related to EBV infection were reported in immunocompromised persons^[10]. Liver injury may be one of the manifestations of CAEBV^[11-13]. However, only limited knowledge exists about EBV-related hepatitis in immunocompetent patients. We analyzed the clinical and laboratory findings in 15 patients with chronic hepatitis and no data of immune deficiency. Based on the serological profile of reactivated EBV infection and the absence of other major etiological agents, we propose reactivated EBV infection as a cause for the chronic liver disease. Furthermore, we demonstrate that the only significant changes at the level of peripheral blood lymphocytes were the prevalence of CD45RA⁺ T cells, combined with expansion of CD8⁺ T cells lacking CD27 and CD28 expression, and an increased percentage of lytic EBV epitope-specific CTL.

MATERIALS AND METHODS

Patients

Fifteen Caucasian HLA-A2+ patients (11 males, 4 females, mean age 40.6 years (range 19-55) with chronic hepatitis, referred to the Clinic of Gastroenterology, Medical Institute, Ministry of Interior, Sofia between Sep 2004 and Mar 2005, were included in this study. Chronic liver injury was detected during routine prophylactic examination and was defined as an elevation of serum aminotransferases of more than 6 mo duration. All patients were HIV-1, HBV and HCV-negative, had normal serum protein levels, and had no evidence of metabolic, or alcohol-related disorders, or drug toxicity (Table 1). Furthermore, they had no history of prior immune deficiency or of any other recent, recurring or chronic infection, or immunosuppressive factors that might explain the observed pathological changes.

A control group included 15 HLA-A2⁺ EBV⁺ sex- and age-matched healthy volunteers with normal aminotransferase levels. All samples were obtained after informed consent.

Serological and routine laboratory tests

EBV viral capsid antigen (VCA) IgG and IgM, EA-R and EA-D IgG and Epstein-Barr nuclear antigen (EBNA) were measured, using immunofluorescent assay (IFA FDA Atlanta kits). HIV-1, HBsAg, anti-HBc and anti-HCV were tested with ELISA method. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (gamma-GT, GGTP), creatinine, bilirubin, glucose levels, serum proteins and coagulation were measured according to standard laboratory procedures.

Immunophenotypic studies

Whole blood was obtained in heparinized vacutainer tubes. Lymphocyte subset absolute counts were determined

Table 1 Exclusion criteria

Alcohol consumption > 40 mL per day
Diabetes or impaired glucose tolerance (including hyperinsulinaemia, OGTT and fasting blood glucose)
Under- or overweight (BMI < 18 or > 26 kg/m ²)
Dyslipidaemia
Hyper- or hypothyroidism
Gluten enteropathy
Autoimmune hepatitis or PBC/PSH
Viral hepatitis B or C
Hereditary hemochromatosis or Iron overload
Wilson disease
Anamnesis for hepatotoxic drugs use (including herbal and over-the-counters in the last 12 mo)
Hereditary muscular disorders

Table 2 T cell subsets analyzed in this study

mAb combination	Subset	Phenotype
CCR7/CD45RA/CD8/CD4	Naïve CD8	CD8 ⁺ CD45RA ⁺ CCR7 ⁺
	Antigen-primed/memory CD8	CD8 ⁺ CD45RA ⁺
	Effector CD8	CD8 ⁺ CD45RA ⁺ CCR7 ⁺
	Naïve CD4	CD4 ⁺ CD45RA ⁺ CCR7 ⁺
	Antigen-primed/memory CD4	CD4 ⁺ CD45RA ⁺
CD27/CD28/CD8	Early CD8	
	Intermediate CD8	CD8 ⁺ CD27 ⁺ CD28 ⁺
	Terminally differentiated CD8	CD8 ⁺ CD27 ⁺ CD28 ⁺

by a lysis/no wash procedure with TruCOUNT tubes and CD3/CD4/CD45/CD8 MultiTest; T cell subsets were evaluated by multicolor immunophenotyping and a standard lysis/wash technique (Table 2). The quantitative expression of CD38 on CD8⁺ T cells (CD38 ABC) was assessed by the QUANTIBRITE PE fluorescence quantitation kit, according to the manufacturer's instructions and analyzed with QUANTIBRITE software. All reagents were products of BD Biosciences (B-D, San Jose, CA). At least 5000 lymphocytes were collected per sample and analysed using FACSCalibur flow cytometer and CellQuest software (B-D).

Intracellular cytokine analysis

The HLA-A2 restricted epitope GLCTLVAML from the EBV lytic cycle protein BMLF1 was synthesized according to the standard Fmoc protocol^[14]. EBV peptide (10 mg/L) or PHA (Sigma) were used for overnight stimulation of 250 µL whole blood (at 37°C and humidified 5% CO₂ atmosphere). Two hours later 12.5 mg/L Brefeldin A (BD) was added. Cells were further washed in cold phosphate buffered saline (PBS), incubated with EDTA for 10 min, washed again and processed according to the protocol for surface and intracellular staining. For intracellular staining, 100 µL of stimulated blood was lysed with 1 mL FACS™ Lysing solution (BD) followed by permeabilization with 0.5 mL 1 × FACS permeabilizing solution (BD) for 10 min. After washing with PBS containing 0.5% BSA, a simultaneous staining for IFN-γ/CD69/CD8/CD4 was performed for 30 min at room temperature in the dark.

Table 3 Characteristics of the patients with chronic hepatitis related to Epstein-Barr virus infection

Pt No.	Age (yr)	Sex	Duration of illness (mo)	AST	ALT	ALP	GGT	Serology EBV (IFA)				
								VCA IgG	VCA IgM	EA-R IgG	EA-D IgG	EBNA
1	40	m	24	52	90	300	200	320	10	80	80	0
2	60	m	6	50	187	280	70	160	0	160	160	40
3	27	m	6	31	107	200	20	640	0	160	160	40
4	40	m	6	30	60	300	40	160	0	160	160	40
5	57	f	24	66	84	280	110	320	0	160	160	40
6	55	f	6	66	85	850	171	160	0	80	80	40
7	28	m	6	40	96	200	60	160	10	80	80	20
8	38	m	12	60	60	150	130	640	0	80	80	20
9	30	m	6	50	125	250	180	640	0	80	80	20
10	25	m	6	20	60	225	90	160	0	80	80	20
11	35	m	6	40	80	136	180	160	0	80	80	40
12	31	m	6	50	80	310	80	320	0	160	160	40
13	43	m	10	60	100	300	100	320	10	160	160	0
14	50	f	6	100	200	130	200	640	0	160	160	40
15	50	f	6	80	120	400	200	320	0	160	160	40

For analysis of intracellular staining at least 25 000 CD8^{high} cells were collected. Detection of at least 0.05% IFN- γ ⁺CD69⁺CD8^{high} cells, after subtraction of non-stimulated control values was considered as significant response.

Liver biopsy and imaging

All patients underwent conventional abdominal ultrasonography. Liver biopsy specimens were obtained from 5 patients.

Statistical analysis

Man-Witney non-parametric test was used to evaluate differences between patient and control groups. *P* values less than 0.05 were considered statistically significant.

RESULTS

Clinical findings

The clinical characteristics of each patient are summarized in Table 3. The estimated mean disease duration before the prophylactic examination was 10.8 (min 6, max 36) mo. At the time of diagnosis of the chronic liver disease none of the 15 patients had an infectious mononucleosis-like illness or any other significant complaints except for one, who reported periods of sub-febrile temperature. None of the patients reported previous opportunistic infections or other significant chronic diseases, or congenital immunodeficiency, or immunosuppressive treatment. The average score of Karnofsky performance status was 95%^[15].

Laboratory tests showed normal blood counts. Mean values (95% CI) are as follows: white blood cell count, $6.61 \times 10^9/L$ (5.945 to 7.281); red blood cells $5.08 \times 10^6/L$ (4.7893 to 5.3707); platelets, $243 \times 10^9/L$ (207.31 to 277.89). The mean range of serum ALT at the time of diagnosis was 103 UI/mL (UNL = 40) (SD 40.9, SE 10.57), AST/ALT ratio < 1, gamma-GT 122 UI/mL (UNL = 48) (SD 62.41, SE 16.12). Normal serum protein and albumin levels were established in all patients, and

no hyperbilirubinaemia or coagulation disturbances were found. Fasting glucose levels, as well as the glucose load test were within the reference ranges. A serum insulin level in 12 patients was below upper limit, while 3 patients were not tested. The abdominal ultrasonography series revealed normal liver and spleen image.

EBV-serology at the time of diagnosis

The EBV-specific antibody titers of each patient are shown in Table 2. Serology tests indicated a reactivated EBV infection in all 15 patients. The average EBV VCA IgG titer was 320 UA/mL (160-640), EBNA average titer was 20 UA/mL (10-40), and EA-R and EA-D were elevated (80-160). EBV VCA IgM was not detected in any patient. All controls were EBV VCA IgM-negative.

Lymphocyte subset analysis

The percentage and absolute counts of total lymphocytes, T, B and NK cells, CD4⁺ T cells as well as the CD4/CD8 ratio were within the established reference ranges for healthy age-matched controls. Using the mAb combinations CD45RA/CCR7/CD8/CD4 and CD27/CD8/CD28, naïve, central and effector memory, as well as terminally differentiated effector T cells (CTL) were defined (Table 2). The fine subset analysis of T cells revealed a significant decrease of naïve (CD45RA⁺CCR7⁺) CD4⁺ ($P < 0.05$) and CD8⁺ ($P < 0.01$) T cells accompanied by an increased percentage of memory (CD45RA⁻) CD4⁺ ($P < 0.01$) and CD8⁺ T cells ($P < 0.001$), as well as of the terminally differentiated (CD28⁻CD27⁻) CTL subset ($P < 0.01$) (Figure 1A-E). A moderately increased number of CD38 molecules on CD8⁺ T cells was established (mean, 2150; min, 1090; max, 4413) as compared to controls (mean, 1522; min, 510; max, 2210; $P < 0.05$), most probably corresponding to a low level viral replication.

Circulating CD8⁺ T cells, expressing IFN- γ in response to the lytic BMLF1 epitope were registered in all patients, and their percentage was significantly increased (15/15; mean, 0.39%), as compared to EBV-seropositive donors

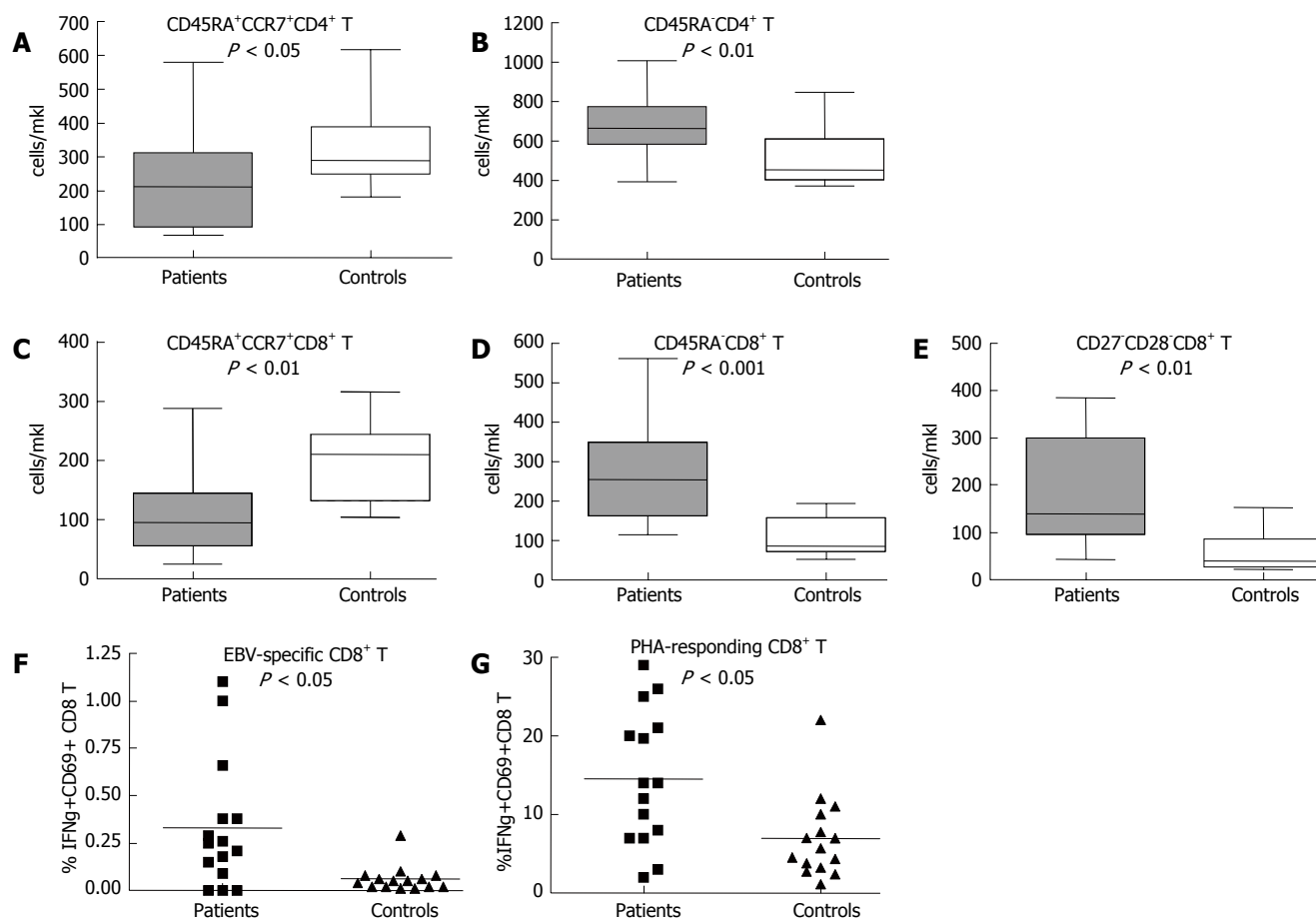


Figure 1 Significant differences detected between patients and control group by immunophenotyping. Patients were characterized by a decreased absolute number of naive CCR7⁺CD45RA⁺CD4⁺ (A) and CD8⁺ (C) T cells, increased absolute number of memory CD45RA⁺CD4⁺ (B) and CD8⁺ (D) T cells, increased absolute number of effector CD28⁺CD27⁺CD8⁺ T cells (E), as well as by increased percentage of IFNγ⁺CD69⁺CD8⁺ T cells after stimulation with EBV peptide GLCTMVL (F) and with PHA (G). Box-and-whiskers indicate the 25-75 percentile and min-max values for each group. Mean values are designated on all plots by a horizontal line. Man-Witney. *P* values are indicated for each comparison.

(6/15; mean, 0.06%; $P < 0.05$). The percentage of PHA-responding CD8⁺ T cells was also significantly increased in the patients group in comparison to controls (14.5% *vs* 7.8%, $P < 0.05$) (Figure 1F and G).

Histopathology

Liver biopsy specimens revealed non-specific histopathologic features of low-grade hepatitis. On routine HE slides mild portal and periportal mononuclear infiltrates were seen, composed of small lymphocytes lacking significant cytologic atypia. According to the METAVIR system all patients were staged as A2. No fibrosis was found in any patient except in one which was graded F1 (Figure 2).

DISCUSSION

EBV infects the majority of the human population and persists in the host organism for life. CD8⁺ T lymphocytes specific for lytic and latent EBV antigens control the virus both during primary infection and the long-term carrier state and almost no symptoms indicate these events^[2,16]. Therefore, the development of cellular immune deficiency, related to HIV-infection, transplantation, prolonged immunosuppressive therapy, or even psychological/physical stress is often combined with complications

due to EBV reactivation^[17]. A broad array of clinical entities has, therefore, been related to EBV infection in immunocompromised host, including persistent and necrotizing hepatitis^[18,19]. CAEBV, characterized by a specific pattern of serological and clinical findings, may also induce liver injury^[11,12].

We reported the clinical and laboratory features of 15 Caucasian non-related patients with chronic hepatitis in the settings of reactivated EBV infection that had no apparent immune deficiency and did not meet the classical criteria for CAEBV infection. The serological evidence for EBV reactivation was the presence of EBV-NA and EA-R antibodies, combined with elevated but not extreme titers of IgG anti-VCA^[20]. None of the patients had significant clinical manifestation of the infection, except for one. Although we did not determine plasma and tissue viral load, the moderately elevated CD38 expression on CD8⁺ T cells suggested rather a low-level of viral replication^[21]. In fact, reactivated EBV infection cases have been reported with mild to moderate clinical presentation, and without extreme EBV-specific Ab titers or viral load^[3]. Our data further suggest that the reactivation of EBV infection in the settings of a functional immune system may be more frequent than previously assessed.

Since the latency of EBV infection depends almost entirely on efficient cellular immune responses, phenotypic

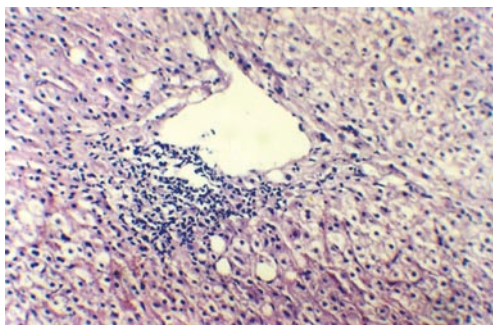


Figure 2 Liver biopsy, showing lymphocyte infiltrate within the lobule and some hepatocyte injury, with mild steatosis displayed in some cells.

and functional changes in the lymphocyte compartment might be expected in case of reactivation. CAEBV is usually characterized by oligoclonal expansions of either T or NK cells^[22,23]. Although we detected no significant changes in the main circulating lymphocyte populations, the fine subset analysis revealed a significant prevalence of antigen-primed (CD45RA⁺) over naïve (CD45RA⁺CCR7⁺) CD4⁺ and CD8⁺ T cells, combined with increased percentage of terminally differentiated (CD28⁺CD27⁺) CD8⁺ T cells. In general, the activation and differentiation state of the circulating T cell pool reflects any major antigen-driven process in the organism. T-cell activation induces a shift from CD45RA to CD45RO expression, while long-lasting chronic viral infections like HIV-1 or CMV are characterized with prevalence of terminally differentiated non-proliferating effectors in the CD8⁺ T cell compartment^[24,25], similar to the natural age-related senescence of cellular immunity^[26]. Moreover, particular viruses may be associated with specific phenotypic changes in latency, as the CMV-driven expansion of CD45RA⁺CD27⁺CD8⁺ T cells^[27]. Logically, such a phenotypic shift must involve both virus-specific and non-specific by-stander activation and differentiation.

The EBV-specific memory pool in asymptomatic carriers is reported to be heterogeneous: CD8⁺ T cells specific for latent epitopes being mostly CD45RA⁺CD28⁺, while those, specific for lytic epitopes, CD45RA⁺/CD45RA⁺CD28⁺CD27⁺^[28]. CD27 is a co-stimulatory molecule promoting the survival of activated CD8⁺ T cells in the absence of CD28^[29], with a limited reexpression capacity. CD27 probably enhances EBV-specific responses, as most of the EBV-specific cytotoxic activity was reported in the CD27⁺ subpopulation^[30]. In our patients group we established a significant expansion of CD8⁺ T cells lacking both CD28 and CD27. We may speculate that in case of long lasting or repeated low-level EBV reactivation, the CD27⁺ pool would be gradually replaced by CD27⁺ CD8⁺ T cells, less efficient in controlling the persistent EBV infection.

EBV-specific cells detected by tetramer staining in latency account for 1%-3% of the circulating CD8⁺ T cell pool^[31]. Again by tetramer staining, a similar detection rate of lytic epitope-specific CD8⁺ T cells has been reported in CAEBV and asymptomatic EBV carriers^[16]. In our series, patients with EBV-related hepatitis had a significantly higher lytic epitope-specific CTL response, as compared to

controls, combined with a higher response to non-specific stimulation, as estimated by IFN- γ expression. These results indicate that the identification of functional EBV-specific CD8⁺ T cells may better differentiate between asymptomatic carriers and clinically unapparent but active infection.

Several viruses have been implicated in the development of chronic liver disease, either as single agents or *via* cross-activation of T cells. A close association between liver lesions and a previous EBV infection has been demonstrated, as well as the presence of EBV-positive cells in affected livers in the settings of continuous low level EBV replication^[32,33]. Sugawara *et al.*^[34-36] linked EBV to HCV-related hepatocellular cancerogenesis, supporting the EBNA-1 promoted HCV replication hypothesis. EBV was suggested to be involved in the hepatocellular carcinogenesis, although this role is still controversial^[37]. Our patients had serological evidence for EBV reactivation and, on the other hand, immunophenotypic evidence for persisting low-level cellular immunity activation and chronic liver inflammation. As the mechanism of EBV-related liver damage remains unclear, indirect, cytokine-induced hepatocellular injury is being discussed^[38,39]. Despite the lack of typical mononucleosis characteristics, liver lymphocyte infiltrates in our cases might be involved in the EBV-provoked immune response.

Although based mostly on exclusion criteria, our data suggest that chronic liver disease could be a manifestation of chronic EBV infection, with persistent low-level virus replication or in case of frequent reactivations. It remains to elucidate the underlying mechanisms of inefficient viral control in these immunocompetent patients and whether such patients may further revert to a fulminant course of the infection.

In conclusion, a reactivated EBV infection might be the possible reason for a chronic elevation of serum aminotransferases without a detectable immune deficiency. The prevalence of memory (CD45RA⁺) and terminally differentiated (CD28⁺CD27⁺) CD8⁺ T cells together with the increased frequency of functionally active EBV-specific CD8⁺ T cells in the circulating CD8⁺ T cell compartment might be the only surrogate markers of reactivated viral infection. The identification and follow-up of such patients may help the prevention of classic CAEBV or other severe manifestations of chronic EBV infection.

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Association of promoter polymorphism of the CD14 C (-159) T endotoxin receptor gene with chronic hepatitis B

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Abstract

AIM: To investigate whether single-nucleotide polymorphisms in the promoter regions of endotoxin-responsive genes CD14 C (-159) T is associated with chronic hepatitis B.

METHODS: We obtained genomic DNA from 80 patients with established diagnosis of chronic hepatitis B and 126 healthy subjects served as a control population. The CD 14 C (-159) T polymorphism was investigated using an allele specific PCR method.

RESULTS: Twenty seven percent of chronic hepatitis B patients and 75% of controls were heterozygous for CT genotype. The difference between the chronic hepatitis B and control groups was statistically significant [$P < 0.0001$; Odds ratio (OR) = 2.887; 95% CI: 1.609-5.178]. Twenty four point six percent of chronic hepatitis B and patients 12.3% of the control group were heterozygous for TT genotype. The difference between groups was not statistically significant ($P = 0.256$; OR = 0.658; 95% CI: 0.319-1.358). Forty eight point four percent of chronic hepatitis B patients and 12.7% of control were homozygote for CC genotype ($P < 0.004$; OR = 0.416; 95% CI: 0.229-0.755). The frequency of allele C was 61.9% and allele T was 38.1% in hepatitis B patients group. The frequency of allele C was 55.2% and allele T was 44.8% for the control group ($P = 0.179$; OR = 1.319; 95% CI: 0.881-1.977).

CONCLUSION: The TT heterozygous genotype was not a risk factor for chronic hepatitis B. CC homozygote genotype is protective for hepatitis B. Lack of heterozygosis of genotype CT is a risk factor for chronic hepatitis B. Alleles C or T were not risk factors for chronic hepatitis B.

These findings show the role of a single-nucleotide polymorphism at CD14/-159 on the development of

INTRODUCTION

An estimated 350 million persons worldwide are infected with hepatitis B virus (HBV). Hepatitis B carriers are at risk for development of cirrhosis and hepatocellular carcinoma. Persons with chronic hepatitis B infection need life-long monitoring to determine when intervention with antiviral therapy is needed and to observe for serious sequels^[1].

The mechanism by which HBV establishes a persistent infection is at present still unclear. Evidence suggests that the clinical manifestations and outcomes after acute liver injury associated with viral hepatitis are determined by the immunologic responses of the host^[2]. CD14, a key gene of the innate immune system, functions as a receptor for lipopolysaccharide (LPS), a constitutive element of the bacterial cell wall. CD14 cannot bind to LPS directly. A protein termed LBP (lipopolysaccharide binding protein) must first bind to LPS. The LPS-LBP complex then binds to CD14 and the receptor-ligand complex is internalized. In addition, CD14 is associated with a protein known as Toll-like receptor 4 (TLR-4). As a consequence of the CD14-LBP/LPS interaction at the level of the membrane, TLR-4 becomes activated. TLR-4 plays an important role in signal transduction. Importantly, TLR-4 is now known to activate a transcription factor known as NF κ B. Viruses have targeted cellular cytokine production and cytokine receptor-signaling pathways, apoptotic pathways, cell growth and activation pathways, MHC-restricted antigen presentation pathways and humoral immune responses^[3].

This study tested whether genetic factors, CD14 C (-159) T, has any role in molecular pathogenesis of chronic

hepatitis B and influences the individual susceptibility for chronic hepatitis B.

MATERIALS AND METHODS

Subjects

We analyzed 80 Iranian patients with chronic hepatitis B and 126 sex-matched control subjects. All chronic hepatitis B patients had visited at a liver clinic in Tehran for regular follow-up examinations. All participants signed the informed consent.

Chronic hepatitis B was based upon biochemical, virologic, histological activity and included patients still on interferon or lamivudine treatment; those who finished treatment course and nonresponders to treatment (lack of virologic and or histologic response by first treatment course in which sustained response was unlikely). Asymptomatic carrier state defined as: chronically HBsAg positive patients who have anti-HBc in serum, anti-HBs is either undetected or detected at low titer against the opposite subtype specificity of the antigen regarded as inactive or asymptomatic carriers, HBV DNA load less than 10^5 copies/mL, HBeAg (+, -), serum liver transaminases of normal range. Inactive hepatitis B surface antigen (HBsAg) carriers were monitored with periodic liver chemistries as liver disease may become active even after many years of quiescence. Controlled subjects had no evidence of hepatitis B infection. The serum of control subjects were evaluated for HBsAg, HBsAb, AST and ALT. Those who had negative HBsAg and HBsAb as well as normal AST and ALT were selected for control groups.

CD14 C (-159) T Genotype Determination

Ten milliliters were collected from each subject into tubes containing 50 mmol/L EDTA, and genomic DNA was isolated from anti-coagulated peripheral blood Buffy coat using Miller's salting-out method^[4].

Total genomic DNA from peripheral blood leukocytes was extracted by standard methods. The CD14 C (-159) T polymorphism was investigated using an allele specific PCR method^[5]. PCR products were visualized by electrophoresis in 2% (w/v) agarose gel stained with ethidium bromide. The assay thus yields a 381-bp band for T allele and 227-bp band for C allele.

Statistical analysis

The differences in the frequencies of the CD14 genotypes and alleles and other risk factors were analyzed by the χ^2 test. For comparing mean stage of liver pathology in different genotypes, Kruskal-Wallis test was used. Associations and differences with probability value less than 0.05 were considered significant. Statistical data was expressed as mean \pm SD. All statistical analyses were performed with the use of SPSS software, version 11.05.

RESULTS

The mean age in hepatitis B group was 36.10 ± 13.78 and in control group was 43.60 ± 16.47 . In the hepatitis B group 71.3% of patients were male and 28.8% were

female. In the control group 62.7% were male and 37.3% were female. The two groups were matched by sex. In the hepatitis B group, 12% were HBeAg positive, 85% were HBeAg negative, 85% were HBeAb positive and 15% were HBeAb negative. Serum aspartate aminotransferase (AST) levels in 75.9% of hepatitis B patients were < 40 IU/mL and in 24.1% were ≥ 40 IU/mL. Serum alanine aminotransferase (ALT) in 68.4% of hepatitis B patients was < 40 IU/mL and in 31.6% was ≥ 40 IU/mL. The state of hepatitis B disease at the time of sampling was as follows: 53.8% were asymptomatic carriers, 31.3% were still on antiviral treatment, 6.3% again returned to treatment, 7.5% were in chronic state and 1.3% were cirrhotics. Liver biopsy was performed in 36 patients of hepatitis B group. Histological classification was measured by Modified Histological activity index (HAI) by Ishak score^[6]. Mean stage, grade and pathologic score were 1.85 ± 0.97 , 5.28 ± 1.84 , and 7.17 ± 2.58 , respectively.

Twenty seven percent of hepatitis and 75% of control subjects were heterozygous for CT genotype. The difference between CT genotype was statistically significant ($P < 0.0001$; OR = 2.887; 95% CI: 1.609-5.178). The lack of heterozygosity for genotype CT was a risk factor for hepatitis B. 24.6% of hepatitis and 12.3% of control group subjects were heterozygous for the TT genotype. The difference between groups was not statistically significant ($P = 0.256$; OR = 0.658; 95% CI: 0.319-1.358). The TT homozygote genotype was not a risk factor for hepatitis B. 48.6% of hepatitis and 12.7% of control group subjects were heterozygous for the CC genotype ($P < 0.004$; OR = 0.416; 95% CI: 0.229-0.755). The CC homozygote genotype was protective for hepatitis B.

The frequency of allele C was 61.9% and allele T was 38.1% in the hepatitis B group.

The frequency of allele C was 55.2% and allele T was 44.8% for control group ($P = 0.179$; OR = 1.319; 95% CI: 0.881-1.977). So alleles were not a risk factor for hepatitis B. There were no statistically significant associations between allele frequencies and genotypes frequencies in the hepatitis B group with state of disease; ALT (< 40 , ≥ 40) IU/mL, mean stage of liver pathology, HBeAg (+, -). Mean stage of liver pathology was not statistically significant in different genotypes (CC, CT, CT) by Kruskal-Wallis test.

DISCUSSION

In a previous study^[7], the effect of recombinant HBsAg (rHBsAg) on LPS- and IL-2-induced activation of monocytes was investigated. It showed that recombinant HBsAg particles, which contain the S protein only, bind almost exclusively to monocytes. Further it showed that recombinant HBsAg (rHBsAg) particles not only inhibit LPS-induced secretion of IL-1 β and TNF α , but also inhibit IL-2-induced secretion of IL-8. Their results suggested that monocytes express a receptor that is recognized by HBsAg and that HBV produces HBsAg in excess amounts to interfere with the normal function of antigen-presenting cells.

In our population, HBeAg negative chronic hepatitis B is more common than HBeAg positive. But difference

in associations of frequency of alleles and genotypes in HBeAg negative versus positive chronic hepatitis B patients were not statistically significant. Besides the viral role, we aimed to investigate the CD14 C (-159) T polymorphism as a host factor, which deteriorates the hepatitis course and outcome in our population.

The CD14 promoter genotype may affect inflammatory processes and be involved in atherogenesis, and it is therefore possible that this genotype might also be associated with other major forms of thrombotic disease, such as ischemic cerebrovascular disease, coronary artery disease. LPS is a structural component of gram-negative bacteria and is bound in plasma by the LPS binding protein^[8]. The LPS binding protein complex then binds to a glycosylphosphatidylinositol-anchored membrane protein, membranous CD14 (mCD14), on monocytes and macrophages and activates these cells. The activated phagocytes in turn secrete inflammatory cytokines through which LPS indirectly activates endothelial cells. Soluble CD14 (sCD14), which lacks a glycosylphosphatidylinositol anchor, can also be found in plasma. Endothelial cells and smooth muscle cells, lacking their own mCD14, are directly activated by LPS-sCD14 complex^[9,10]. Directly and indirectly activated endothelial cells express cell adhesion molecules and increased procoagulant activity, and they release free radicals, thereby mediating the initiation and development of atherosclerosis.

A previous study^[11], demonstrated T allele frequency was significantly higher in myocardial infarction survivors and that the density of monocyte mCD14 was higher in T/T homozygotes than in other genotypes.

In another study^[12], the possible association between the C (-260)→T polymorphism in the CD14 promoter and the occurrence of symptomatic ischemic cerebrovascular disease (CVD) was tested. They concluded that the C (-260)→T polymorphism in the CD14 promoter is not associated with an increased risk for CVD.

A previous study^[13] mentioned activated Kupffer cells release proinflammatory cytokines, a process that is regulated by the CD14 endotoxin receptor (CD14). Also, both clinical and experimental data suggest that Kupffer cell activation by gut-derived endotoxins and other bacterial products is an important pathogenic factor. In that study, the association of CD14 promoter polymorphism with different forms of alcoholic liver damage (ALD) was examined in 3 separate autopsy series. The overall age-adjusted risk for cirrhosis was 3.08 for the carriers of the CT genotype, and 4.17 for the homozygous TT genotype. Their results suggested that in the relatively isolated Finnish population, the T allele and in particular, TT homozygotes confers increased risk of alcoholic liver damage and are at a high risk to develop cirrhosis.

Another study^[14] investigated whether single-nucleotide polymorphisms in the promoter regions of endotoxin-responsive genes CD14 and tumor necrosis factor- α (TNF- α) were associated with biliary atresia (BA) and idiopathic neonatal cholestasis (INC) in 90 patients with established diagnosis of BA and 28 patients with INC. Also, forty-two adult patients with hepatitis B-related cirrhosis and 143 healthy children served as control populations. According to that study the single-nucleotide polymorphism at

CD14/-159 is associated with the development of BA and INC. They concluded that endotoxin susceptibility might play a role in the pathogenesis of infantile cholestasis.

In the present study, we did not compare risk factors of hepatitis B infection, its routes of exposure and transmission in hepatitis B and control groups. Although determining the exact time of hepatitis B infection was impossible. Whether specific polymorphisms of CD14 C (-159) T has any correlation with progression of liver pathology or whether it indirectly produces needs further study and a larger sample size.

CONCLUSIONS

Our study demonstrated that the role for CD14 C (-159) T polymorphism on the development of chronic hepatitis B. Although the number of subjects in the chronic hepatitis B subtypes was relatively small, differences in the genotype distributions for chronic hepatitis B subtypes was significant.

Lack of heterozygosis of genotype CT is a risk factor for chronic hepatitis B.

TT homozygote genotype was not a risk factor for chronic hepatitis B. CC homozygote genotype is protective for chronic hepatitis B.

These findings show a role for single-nucleotide polymorphisms at CD14/-159 C (-159) T on development of chronic hepatitis B. Endotoxin susceptibility may play a role in the pathogenesis of chronic hepatitis B.

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Inhibition of hepatitis B virus production by *Boehmeria nivea* root extract in HepG2 2.2.15 cells

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Abstract

AIM: To explore the anti-hepatitis B virus (HBV) effects of *Boehmeria nivea* (*B. nivea*) root extract (BNE) by using the HepG2 2.2.15 cell model system.

METHODS: Hepatitis B surface antigen (HBsAg), hepatitis B virus e antigen (HBeAg), and HBV DNA were measured by using ELISA and real-time PCR, respectively. Viral DNA replication and RNA expression were determined by using Southern and Northern blot, respectively.

RESULTS: In HepG2 2.2.15 cells, HBeAg (60%, $P < 0.01$) and particle-associated HBV DNA ($> 99\%$, $P < 0.01$) secretion into supernatant were significantly inhibited by BNE at a dose of 100 mg/L, whereas the HBsAg was not inhibited. With different doses of BNE, the reduced HBeAg was correlated with the inhibition of HBV DNA. The anti-HBV effect of BNE was not caused by its cytotoxicity to cells or inhibition of viral DNA replication and RNA expression.

CONCLUSION: BNE could effectively reduce the HBV production and its anti-HBV machinery might differ from the nucleoside analogues.

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Key words: *Boehmeria nivea*; Medicinal herb; Antiviral agent; Hepatitis B virus; Anti-hepatitis B virus; HepG2 2.2.15

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INTRODUCTION

Hepatitis B virus (HBV) has infected more than 4 million people worldwide. About 20% of patients infected with HBV may lead to chronic hepatitis, liver cirrhosis, and hepatocarcinoma. HBV, belonging to the *Hepadnaviridae* family, is a non-cytopathic DNA virus with an icosahedral capsid that replicates *via* reverse transcription of an RNA intermediate^[1]. Although the molecular biology aspects of the HBV genome have been described in detail, the mechanisms of viral packaging and transport remain to be elucidated^[2]. Nevertheless, HBV-DNA-transfected cells and virus infection animal models have aided the efforts to reveal the mechanism behind the HBV replication cycle. These results led to the identification of the first antiviral agents targeting the reverse transcription process^[3].

Chronic hepatitis type-B patients are clinically treated with interferon alpha (INF- α) and nucleoside analogue lamivudine (3TC), adefovir or entecavir^[4,5], which are analogues of reverse-transcriptase inhibitors^[6,7]. INF- α inhibits viral replication and acts as an immuno-modulator. However, its disadvantages include limited effectiveness (40% response rate)^[8], low efficacy with respect to cost, and serious side effects. For 3TC, its inhibition is reversible, and continuous treatment often leads to the development of drug-resistant HBV variants (70% of patients after 4 years of treatment)^[9]. Both adefovir and entecavir are used against 3TC-resistant viruses; however, resistances to these two drugs have been reported in lamivudine-resistant patients^[10-12]. The use of combination therapy, such as INF- α plus 3TC, 3TC plus adefovir and 3TC plus entecavir, may yield additive or synergistic effects or reduce the emergence of resistance, though serious side effects and unsatisfactory efficacy still present problems. Undeniably, there is a demand for new and improved therapies.

The large repertoire of herbal compounds may show potential in developing new ways to combat previously considered "incurable" diseases, provided that these compounds (or often, mixtures of compounds) could satisfy current government regulations. At present, alternative or traditional medical resources are used by more than 80% of the population in developing countries and by an increasing number of people in other parts of

the globe^[13,14]. Complementary and alternative therapies for chronic hepatitis are also intensively explored and the results appear promising^[15]. Patients with chronic liver diseases are treated with some medicinal herbs exhibiting strong anti-viral activities^[16], including daphnoretin from *Wikstroemia indica*^[17], costunolide and dehydrocostus lactone from *Saussurea lappa* Clarks^[18], osthole from *Angelica pubescens*^[19], and the extracts of genus *Phyllanthus* of the *Euphobiaceae* family^[20]. Furthermore, genus *Phyllanthus* exhibited a positive effect on the clearance of serum HBsAg in clinical trials conducted on chronic HBV infections, and a synergistic effect when administered with IFN- α ^[20]. *B. nivea* has been distributed and used therapeutically in China and Taiwan for diuretic, antipyretic, and hepatoprotective purposes. Recently, it has been reported that root extracts of *B. nivea* exhibited hepatoprotective activities against CCl₄-induced liver injuries, and anti-oxidant effects on FeCl₂-ascorbate-induced lipid peroxidation in rat liver homogenate^[21].

To investigate the anti-viral mechanism of *B. nivea* extract (BNE), HBV-producing hepatoma HepG2 2.2.15 cells, which secrete HBsAg, HBeAg and complete Dane particles^[22], were chosen for the evaluation of the anti-HBV effect of BNE. Here, we assess anti-HBV activities of BNE by measuring HBsAg, HBeAg, HBV DNA in supernatant, and replication intermediate HBV DNA and HBV RNA within the cells.

MATERIALS AND METHODS

Preparation of BNE

To prepare the *B. nivea* plant extract utilized in our experiments, the roots of the plants were collected and dried. One hundred gram of the dried roots was cut into pieces approximately 0.5 cm in length before boiling them in 1 L of 200 mL/L ethanol (1:10 ratio) under reflux for 3 h. The decoction was filtered through a 0.22- μ m filter and lyophilized. The lyophilized powder was dissolved in normal PBS and adjusted to stock concentration (100 g/L) prior to application to the cells.

Cell lines and culture

The HepG2 2.2.15 cell line was kindly provided by Dr. Ho MS (Academia Sinica, Taipei, Taiwan, China), and Human hepatoma HepG2 cells were obtained from American Type Culture Collection (ATCC). These cells were maintained in MEM (Eagle) plus 100 mL/L fetal bovine serum (FBS) supplement with 1.5 g/L sodium bicarbonate, 0.1 mmol/L non-essential amino acids, 1.0 mmol/L sodium pyruvate, and 100 units/mL penicillin G and 100 mg/L streptomycin. A final concentration of 200 mg/L G418 was contained in the medium for the maintenance of HepG2 2.2.15 cells. Before the experiment, the cell count was adjusted to 1×10^6 /mL, and cell viability to higher than 85% by trypan blue exclusion test.

Drug treatment protocols

For drug treatment, 1×10^5 cells of HepG2 2.2.15 were seeded in a 12-well plate and allowed to grow for 3 d before treatment with different concentrations of BNE. Cells were refed with drug-containing fresh medium every 3 d for up to 12 d in time-dependent experiment. In Southern and

Northern blot assay, the culture condition was the same as described above (10 mg/L 3TC were also added), and in which time of drug treatments were only for 1 d.

Analysis of cytotoxicity

HepG2 2.2.15 cells were used for determining cytotoxicity of BNE. Cells were inoculated onto a 96-well plate at a density of 1×10^4 cells per 100 μ L prior to drug treatment. BNE was added at concentrations of 0.1, 1, 10, and 100 mg/L and the cells were refed with drug-containing fresh medium every 3 d for up to 12 d. After drug treatment, the cytotoxicity was measured based on the reduction of MTT (Sigma, St. Louis, USA) in mitochondria^[23].

Determination of HBsAg and HBeAg levels by ELISA

Conditioned medium was collected and the HBsAg and HBeAg levels were determined semi-quantitatively using ELISA assay [SURASE B-96 (TMB), General Biologicals corp., Taiwan, China] according to the manufacturer's instructions.

Supernatant HBV DNA extraction and analysis by quantitative real-time PCR

The supernatant HBV DNA was extracted from conditioned medium as previously described^[24] and stored in -20°C prior to real-time PCR analysis. The quantity of HBV DNA in culture medium was quantified with the ABI 7500 Sequence Detection System by using HBV RealQuant PCR kit (General Biologicals Corp., Taiwan, China) according to the manufacturer's instructions. Briefly, the PCR programming was performed with an initial denaturing steps at 50°C for 2 min and 95°C for 10 min, followed by 45 amplification cycles at 95°C for 15 s and annealing/extending at 58°C for 1 min.

The 50% effective concentration (EC₅₀), defined as the drug concentration that reduces the level of HBV DNA in the culture medium by 50%, was calculated by four parameter logistic curve equation.

Southern and Northern blot detection of HBV DNA and mRNA in HepG2.2.15 cell

HepG2 2.2.15 cells were cultured in MEM medium and treated with 0.1, 1, and 10 mg/L BNE for 1 d. Cells were lysed with 0.8 mL of 0.01 mol/L Tris-HCl (pH 8.0), 0.05 mol/L NaCl, 5 mL/L NP-40, 1 mmol/L EDTA at room temperature for 10 min as previously described^[25]. For the Southern hybridizations, 20 μ g of total DNA was digested with *Hind*III, electrophoresed on a 14 g/L agarose gel, and then transferred to nylon membrane. The probe for hybridization was synthesized from PCR amplification of a plasmid containing the full-length HBV genome (kindly provided by Dr. Ho MS, Academia Sinica, Taipei, Taiwan, China), and then the probe was labeled with digoxigenin-dUTP (DIG) by the DIG-high Prime DNA labeling and Detection Starter kit II according to the manufacturer's protocol (Roche, Basel, Switzerland). The membrane was hybridized with HBV probe at 50°C overnight. For Northern blot analysis, total RNA was isolated from BNE-treated and untreated HepG2 2.2.15 cells by using the TRIZOL kit (Invitrogen, CA, USA). A total of 20 μ g of RNA was resolved in 12 g/L denatured gel and then

transferred onto the nylon membrane and the membrane was hybridized with DIG-labeled HBV DNA fragment described above. For hybridization of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), the full-length HBV DNA probe was removed from the membrane by washing twice at 37°C in 0.2 mol/L NaOH containing 1 mL/L sodium dodecyl sulfate (SDS) solution for 15 min and then re-hybridized with DIG-labeled probe for G3PDH.

Statistical analysis

Data were expressed as mean \pm SE of three independent experiments. Statistical analysis was performed using Student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Effects of BNE on secreted HBsAg, HBeAg and HBV DNA from HepG2 2.2.15 cultures

Anti-HBV activity of BNE was investigated by using HepG2 2.2.15 cells, which can secrete HBV particles. When HepG2 2.2.15 cells were treated with various concentrations of BNE, secretion of HBeAg, but not HBsAg, was significantly suppressed compared to vehicle controls (Figure 1A and B). The suppression of HBeAg was dose-dependent and approximately 20% of inhibition was observed in cells treated with 10 mg/L BNE. Moreover, a significant suppression of HBeAg secretion (approximately 60%) was observed in cells treated with 100 mg/L BNE (Figure 1B).

We also measured the amount of viral DNA secreted in the medium (supernatant HBV DNA) by using real-time PCR. As shown in Figure 1C, supernatant HBV DNA was dramatically decreased compared to vehicle control, since the first day of treatment, in 10 and 100 mg/L BNE-treated culture medium. Although the amounts of supernatant HBV DNA increased while cells continued to grow during the experiment, approximately 95% inhibition of DNA secretion was observed in the 12-d culture treated with BNE (10 mg/L) (Figure 1C). Notably, these results showed that the production of HBsAg and HBeAg was only slightly suppressed by 10 mg/L BNE, but supernatant HBV DNA levels were dramatically suppressed by BNE at the same dose.

Cytotoxic effects of BNE on HepG2 2.2.15 cells

Suppression of HBV production by BNE might be a result of its cytotoxicity, and this possibility was examined in HepG2 2.2.15 cells by using MTT assay. No apparent cytotoxicity was detected in HepG2 2.2.15 cells up to 12 d after exposure to BNE at different concentrations (0.1, 1, 10 and 100 mg/L) (Figure 2), suggesting that the suppression of supernatant viral DNA levels by BNE was not caused by its cytotoxicity.

Determination of the effective concentration of BNE anti-HBV activity

The effective concentration of BNE to suppress 50% of secreted HBV DNA (EC_{50}) was determined with various concentrations of BNE on d 1. The suppression of secreted HBV DNA was shown in a dose-dependent

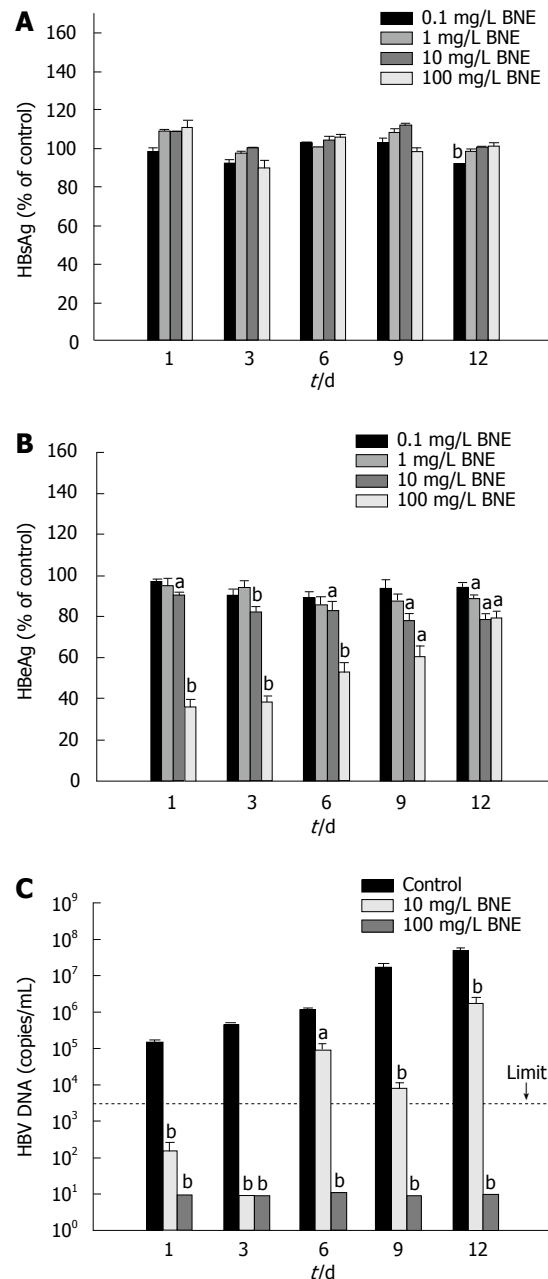


Figure 1 Effects of BNE on secreted HBsAg, HBeAg and HBV DNA from HepG2 2.2.15 cell cultures. A: HBsAg level; B: HBeAg level; C: Viral DNA. The dotted line presents the limitation of this kit (3000 copies/mL). Data are expressed as mean \pm SE of three independent experiments. ^a*P* < 0.05; ^b*P* < 0.01 vs the corresponding controls (Student's *t*-test).

manner with BNE treatment (70% at 0.1 mg/L and 100% at \geq 10 mg/L) (Figure 3); accordingly, the EC_{50} was 0.0462 mg/L. Here, the concentration of BNE (10 mg/L) used for the study of anti-HBV activity was about 200-fold of EC_{50} .

Effects of BNE on intracellular HBV DNA replication and transcription in HepG2 2.2.15 cells

To address the mechanism of inhibition by BNE in HepG2 2.2.15 cells, we analyzed the viral mRNA after 24 h exposure to BNE by Northern blot using full-length HBV genome as a probe (Figure 4A) and intracellular relaxed circular (RC) and single-stranded (SS) forms of HBV DNA by Southern blot in parallel (Figure 4B). The levels

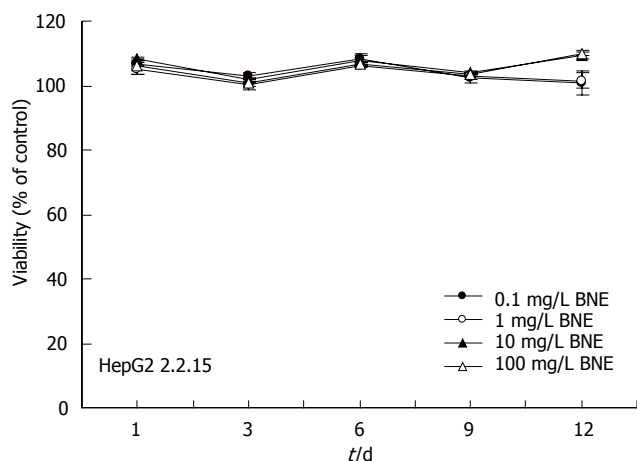


Figure 2 Cytotoxic effects of BNE on HepG2 2.2.15 cells. Data are expressed as mean \pm SE of three independent experiments (MTT assay).

of viral mRNA were not affected in either BNE-treated or 3TC-treated cells (Figure 4A). Moreover, Southern blot results indicated that BNE did not suppress intracellular RC and SS forms of HBV DNA. In Contrast, an apparent inhibition of intracellular RC and SS forms of HBV DNA was observed in 3TC-treated cells, which was significantly different from the BNE group (Figure 4B). These results suggested that BNE did not apparently decrease viral DNA replication and viral mRNA expression in HepG2 2.2.15 cells, and that the anti-HBV mechanism of BNE seemed to be different from that of 3TC.

DISCUSSION

In this study, we first demonstrated that BNE had anti-HBV activity of inhibiting the supernatant HBV DNA levels in a dose-dependent manner in HepG2 2.2.15 cells without blocking HBsAg secretion. This inhibition was caused neither by the toxicity of BNE to HepG2 2.2.15 cells, nor by blocking HBV gene expression and replication. The significant inhibition of supernatant HBV DNA levels was observed at a concentration greater than 10 mg/L of BNE in HepG2 2.2.15 cells. In addition, BNE could also dose-dependently inhibit the secretion of HBeAg. Although BNE had higher inhibitory ability on HBeAg secretion at 100 mg/L than that at 10 mg/L, it still could efficiently inhibit secreted HBV DNA at the latter concentration. This result might be due to the fact that BNE comprise multiple compounds, and the effective concentration of those compounds to inhibit secreted HBV DNA was lower than that of the compounds required for inhibition of HBeAg.

Currently, 3TC and other nucleoside analogues have been shown to inhibit HBV replication both *in vitro* and *in vivo*^[24,26,27]. A previous study had reported that 3TC, a viral polymerase inhibitor, reduced episomal DNA (RC and SS form), whereas HBV-specific RNAs were not affected in HepG2 2.2.15 cells^[24]. In this study, we could not find any apparent reduction of HBV-specific RNAs or intracellular SS and RC DNA in response to BNE, and that these data revealed that the anti-HBV mechanism of BNE might be different from 3TC that targeted the viral

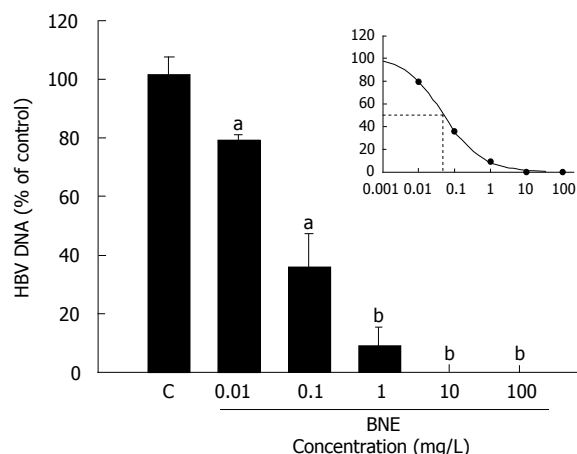


Figure 3 Determination of the effective concentration of BNE anti-HBV activity. Cells were treated for 24 h. Data are expressed as mean \pm SE of three independent experiments. ^a $P < 0.05$, ^b $P < 0.01$ vs the corresponding controls (Student's *t*-test).

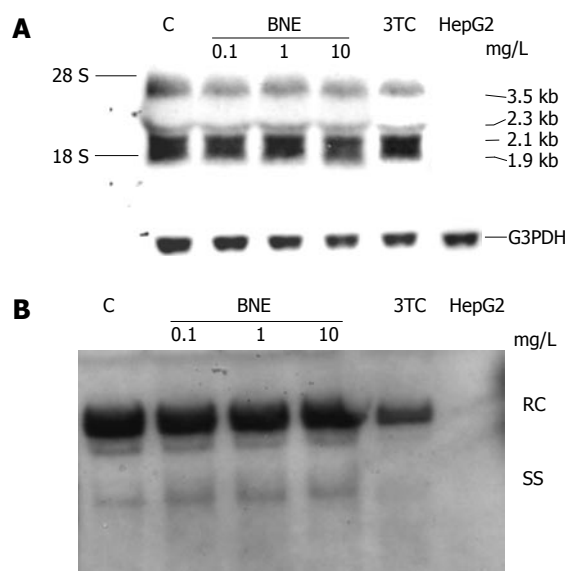


Figure 4 Effects of BNE on intracellular HBV DNA replication and transcription in HepG2 2.2.15 cells. Cells were treated for 1 d. **A:** Northern analysis; **B:** Southern analysis.

polymerase. Since the HBV mRNAs were transcribed from the integrated DNA, it was not unexpected that HBV-specific transcripts were not affected by BNE treatment. The finding that supernatant HBV DNA rather than HBsAg and HBeAg was dramatically inhibited by 10 mg/L BNE after 24-h treatment might come from the possibility that exported virions have outer protein coats or HBsAg without packaging DNA. Though the mechanism of anti-viral effects by BNE remains unclear, we deduced that BNE might block coating and secretion of HBV containing nucleocapsids or destabilize HBV DNA containing nucleocapsids.

Whether the HBV-inhibiting effects of BNE could be contributed to a single component, or multiple components, is currently unknown. In order to explore the active compound for anti-HBV activity, we have attempted to analyze the chemical composition of BNE by HPLC. Very

little, if any, nucleotide analogues were detected (data not shown). Fractionation experiments of BNE are currently being executed in our laboratory. Recently, Herteroaryldihydropyrimidines (HAP)^[28], Bis-ANS^[29], and alkylated imino sugars^[30,31] have been found to block the viral production by interference with either nucleocapsid assembly or nucleocapsid maturation, and these compounds might have the potential to be developed as non-viral polymerase targeting antiviral drugs.

In conclusion, the BNE, together with other medicinal herbs that exploit different action modes to inhibit HBV, could be administered in combination with other polymerase inhibitors or cytokines, providing possibly a novel HBV treatment strategy to the current therapies.

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RAPID COMMUNICATION

Resection of non-cystic adenocarcinoma in pancreatic body and tail

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INTRODUCTION

The prognosis of pancreatic body and tail cancer is dismal^[1-10]. Its curative resection is rarely performed, only a few reports are available^[4-10]. The outcome of 8 Chinese patients with non-cystic adenocarcinoma in pancreatic body and tail (NCAPBT) after resection is reported and the clinical-pathological characteristics and surgical strategy of NCAPBT are discussed in this paper.

MATERIALS AND METHODS

Resection of NCAPBT was performed in eight Chinese patients with complete clinical-pathological data from January 2000 to September 2004 in our hospital. The outcome of all the patients is summarized in Tables 1 and 2. The average age of the patients was 67 years (range, 53-79 years) and the ratio of male to female was 1:1. Abdominal pain was found in 87.5%, weight loss in 75% and back pain in 62.5% patients, respectively. No patient had jaundice. The mean tumor size was 5.04 cm (range, 2.3-8 cm). Curative distal pancreatectomy and splenectomy were performed in 3 patients, palliative distal pancreatectomy and splenectomy in 4 patients, palliative segmental pancreatectomy in 1 patient. Curative resection was defined as resection with all gross tumor tissues removed and negative microscopic margins. Resection with gross residual tumor and/or distal metastasis was considered palliative resection. All the 8 patients had histology-verified NCAPBT. Seven of 8 NCAPBTs were ductal adenocarcinoma. No.4 NCAPBT was mucinous non-cystic adenocarcinoma (Tables 1 and 2). No cystic adenocarcinoma and other rare adenocarcinomas such as adenosquamous carcinoma, undifferentiated adenocarcinoma and primary cancer were found. All the 8 patients were followed up. The log-rank test was used for comparison of differences in survival. Chi-square test or Fisher's exact test was used to evaluate the correlation between categorical data. $P < 0.05$ was considered statistically significant.

RESULTS

The outcome of the 8 patients is summarized in Tables 1 and 2. Intra-abdominal abscess was found in 1 patient and no major postoperative complications were found in

Abstract

AIM: To report the outcome of Chinese patents with non-cystic adenocarcinoma in pancreatic body and tail (NCAPBT) after resection and to discuss its surgical strategy.

METHODS: Resection of NCAPBT was performed in eight Chinese patients with complete clinical-pathological data in our hospital from January 2000 to May 2004. The surgical strategy was explored by analyzing the results of these patients.

RESULTS: The resection rate of NCAPBT in patients without back pain was higher than that in patients with back pain (66.67% vs 20%, 2/3 vs 1/5). The prognosis in the group receiving palliative resection was poorer than that in the group receiving curative resection. The median survival time was 12 mo in the curative resection group and 6 mo in the palliative resection group, respectively.

CONCLUSION: The overall survival time of the Chinese patients with NCAPBT is dismal. The Chinese patients after curative resection of NCAPBT have a longer survival time. The Chinese NCAPBT patients with back pain trend to have a lower curative resection rate, but back pain should not be considered a contraindication for curative resection.

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Key words: Pancreas; Adenocarcinoma; Pancreatectomy; Survival; Back pain

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Table 1 Clinical data of NCAPBT

Patient No.	Gender	Age (yr)	Abdominal pain	Back pain	Weight loss (kg)	Location	Size (cm)	Survival (mo)
1	M	79	Y	Y	NA	BT	5	6
2	F	62	Y	N	5	BT	5	6
3	F	66	Y	Y	7.5	BT	6	4
4	M	65	Y	Y	NA	B	5	8
5	M	53	Y	Y	5	BT	NA	6
6	F	71	Y	N	4.5	B	2.3	12
7	F	68	Y	Y	5	BT	4	28
8	M	72	N	N	3	T	8	8

NA: Not available; BT: Pancreatic body and tail; B: Pancreatic body; T: Pancreatic tail; Y: Yes; N: No.

Table 2 Data of surgery and pathology of NCAPBT

Patient No.	pT	pN	pM	Histological differentiation	Resection	Blood loss (mL)	Operative duration (min)	Reason for non-curative resection
1	4	x	0	Moderate	Palliative	600	140	Invasion of super mesenteric artery
2	4	1	0	Well	Palliative	600	235	Adhesion of abdominal aorta
3	4	x	0	Well	Palliative	200	110	Invasion of celiac trunk and abdominal aorta
4	4	x	0	Poor	Palliative	400	185	Invasion of super mesenteric artery
5	4	x	1	Well	Palliative	600	165	Liver metastasis
6	2	0	0	Poor	Curative	100	165	
7	2	0	0	Moderate	Curative	2300	225	
8	2	1	0	Moderate	Curative	600	205	

the other 7 patients. Major complications were defined as those threatening the life potentially. Neither re-operation was performed nor death of patients occurred during operation. None of the 8 patients was treated in ICU. The curative resection rate of the patients without back pain was higher than that of those with back pain (66.67% *vs* 20%, 2/3 *vs* 1/5). The median survival time was 6 in the palliative resection group and 12 mo in the curative resection group, respectively. The latter group had a longer survival time than the former group, while the former group had a higher 1-year survival rate than the latter group (66.7% *vs* 0%).

DISCUSSION

The prognosis of pancreatic body and tail cancer is poor even after surgical resection^[1-10]. Due to the absence of painless obstructive jaundice and earlier symptoms, most patients with pancreatic body and tail cancer are found in an advanced stage, which results in a low resection rate^[1-10]. The 5-year survival rate of pancreatic body and tail cancer patients after surgical resection ranges from 0% to 25%, and the median survival time is 10 to 15.9 mo^[4-10]. In our study, no NCAPBT patient survived longer than 5 years, the median survival time of the patients after curative resection was comparable with that of other reports^[5-10], but the median survival time of the patients after palliative resection was much shorter than that of those after curative resection (Table 2). The portal vein and/or superior mesenteric vein resection combined

with pancreatectomy can be successfully performed^[9,10], preoperative CT scan or other image analysis should be emphasized on the relationship between the tumor and its major adjacent vessels, such as superior mesenteric artery, abdominal aorta and celiac trunk, because they help surgeons to more precisely judge the resectability of the tumor before operation.

Bathe *et al*^[11] reported that old NCAPBT patients have a higher ratio of major postoperative complications and a significantly shorter survival time, and need to receive radical resection. An interesting finding in our study is that three old patients (≥ 70 years of age) had no major postoperative complications, however, 1 of 5 patients (< 70 years of age) had major postoperative complications. No patient received re-operation. Furthermore, 2 patients in the older patient group (≥ 70 years of age) after curative resection had a longer survival time than those after palliative resection, suggesting that surgical resection of NCAPBT in old patients might not result in more major postoperative complications than in younger patients.

Pancreatic cancer patients with back pain have a low resection rate and poor prognosis^[12]. In our study, the patients without back pain tended to have more chances of curative resection, however, the longest survivors were those with back pain, indicating that back pain should not be considered a contraindication, though the patients with back pain had less chance of curative resection.

Metastasis occurs in most patients with adenocarcinoma of the body and tail of the pancreas^[3,10,13,14]. In our study, one patient with liver metastasis had a comparable

survival time with the other 4 after palliative resection, suggesting that the prognosis of patients with liver metastasis after palliative resection of NCAPBT is not poorer than that of patients without distal metastasis.

Sboup *et al*^[10] reported that the survival rate is correlated with histological differentiation. An interesting finding in our study is that three patients with poorly- or moderately-differentiated tumor in the curative resection group had a longer survival time than the other three patients with well-differentiated tumor in the palliative resection group, indicating that though histological differentiation is related with survival, curative resection of NCAPBT is a more important prognostic factor.

Segmental pancreatectomy is mainly performed for benign or low malignant tumor in the pancreatic body and leads to more postoperative complications than distal pancreatectomy^[15]. In our study, one patient after palliative segmental pancreatectomy had no major complications and was discharged ten days after the operation. The survival time was a little longer than that of the other 4 patients after palliative resection, suggesting that segmental pancreatectomy is a feasible surgery and can preserve more pancreatic tissues.

Sboup *et al*^[10] reported that if extended resection is considered necessary for curative resection of the tumor, the resection is justified in patients with NCAPBT. Fabre *et al*^[4] found that only patients with tumor not more than 4 cm in diameter, but without lymph involvement and metastasis have a significant longer survival time after resection. In our study, the patients in the curative resection group had a longer survival time than those in the palliative resection group. The median survival time of the latter group was only half of the former group. No patients survived more than 1 year in the palliative resection group. The data showed that curative resections of NCAPBT should be recommended. Furthermore, in the curative resection group, one patient with a tumor over 4 cm in diameter and lymph involvement, survived only 8 mo which was a little longer than the median survival time of those in the palliative resection group, while survival time of the two patients with tumor not more than 4 cm in diameter but without lymph involvement and metastasis was not less than 1 year, indicating that the theory of Fabre *et al*^[4] seems to fit for the Chinese patients.

In conclusion, the overall survival of Chinese NCAPBT patients is dismal. Chinese NCAPBT patients after curative resection may have a longer survival time. Chinese NCAPBT patients with back pain, tend to have a lower curative resection rate, but back pain should not

be considered a contraindication for curative NCAPBT resection.

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Hepatic intra-arterial infusion of yttrium-90 microspheres in the treatment of recurrent hepatocellular carcinoma after liver transplantation: A case report

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Abstract

Hepatocellular carcinoma (HCC) recurs with a reported frequency of 12%-18% after liver transplantation. Recurrence is associated with a mortality rate exceeding 75%. Approximately one-third of recurrences develop in the transplanted liver and are therefore amenable to local therapy. A variety of treatment modalities have been reported including resection, transarterial chemoembolization (TACE), radiofrequency ablation (RFA), ethanol ablation, cryoablation, and external beam irradiation. Goals of treatment are tumor control and the minimization of toxic effect to functional parenchyma. Efficacy of treatment is mitigated by the need for ongoing immunosuppression. Yttrium-90 microspheres have been used as a treatment modality both for primary HCC and for pre-transplant management of HCC with promising results.

Twenty-two months after liver transplantation for hepatitis C cirrhosis complicated by HCC, a 42-year old man developed recurrence of HCC in his transplant allograft. Treatment of multiple right lobe lesions with anatomic resection and adjuvant chemotherapy was unsuccessful. Multifocal recurrence in the remaining liver allograft was treated with hepatic intra-arterial infusion of yttrium-90 microspheres (SIR-Spheres, Sirtex Medical Inc., Lake Forest, IL, USA). Efficacy was demonstrated by tumor necrosis on imaging and a decrease in alpha-fetoprotein (AFP) level. There were no adverse consequences of initial treatment.

INTRODUCTION

Strict criteria developed to select patients with hepatocellular carcinoma (HCC) for liver transplantation have increased the survival and decreased the recurrence of tumor after transplantation^[1]. However, tumor recurrence and subsequent mortality continue to be observed. Tumor recurrence is reported to occur with a frequency of 12%-18%^[2-4]. Seventy percent of recurrences are observed within the first year after transplantation^[3]. Unfortunately, recurrence-related mortality is high and exceeds 75%^[2]. Extra-hepatic recurrence is encountered most commonly, with an estimated incidence of 67%^[2]. Recurrence isolated to the transplant liver is relatively infrequent, yet, it represents an opportunity for targeted treatment. Local therapies include resection, trans-arterial chemoembolization (TACE), radiofrequency ablation (RFA), ethanol ablation, cryoablation, and external beam irradiation. Treatment efficacy varies according to tumor characteristics such as location, size, and number. Efficacy also varies with the degree of underlying hepatic dysfunction. Hepatic arterial infusion of microspheres impregnated with the beta-radiation emitter yttrium-90 has been successfully employed in the palliative treatment of unresectable HCC and most recently to down-stage HCC to allow liver transplantation^[5]. Results for the treatment of primary HCC reveal a 38%-65% partial response rate, and a median survival of 23 mo which is 2.6-4.7 times the length of median survival seen in historic controls^[6].

The following case report describes a 42-year old man who underwent liver transplantation for hepatitis C

cirrhosis with stage II HCC and 22 mo later developed recurrent HCC isolated to the liver allograft. When conventional treatment modalities failed, hepatic arterial infusion of yttrium-90 beads (SIR-Spheres, Sirtex Medical Inc., Lake Forest, IL, USA) was safely employed to treat the HCC recurrence in the liver allograft.

CASE REPORT

This hispanic man was diagnosed at 38 years of age with end-stage liver dysfunction secondary to hepatitis C infection (genotype 1a) and listed with UNOS for transplantation in 2001 (UNOS status 3, MELD score 12). Nine months after being listed, serial CT examination of the liver suggested a new 1.5 cm lesion in segment IV. Follow-up imaging five months later showed the lesion to have grown in size to 3 cm consistent with HCC (stage II, T2NxMx). AFP level at this time was 38.7 $\mu\text{g/L}$. He underwent percutaneous RFA of the lesion while awaiting transplantation with a slight decrease in the AFP level to 32.9 $\mu\text{g/L}$ and no residual tumor on CT after treatment. He received the standard MELD exception score at the time of 29 for his stage II HCC. A deceased donor liver transplant became available 5 mo after receiving his exception score (3.5 mo after RFA and 23 mo after being originally listed for transplant). The donor was a 52-year old CMV-positive man. Standard caval interposition technique without veno-venous bypass was used and the total graft ischemic time was 7 h and 40 min. Induction immunotherapy consisted of 20 mg of basiliximab, 300 mg of azathioprine and 1 g of methylprednisolone. Contrary to the post-RFA CT scan from 10 wk earlier, pathologic evaluation of the hepatic explant was notable for the presence of three foci of HCC in the right lobe of the liver. The lesions measured 2.8 cm, 2.7 cm and 1.5 cm each, in maximum dimension. The largest was 60% necrotic as a result of RFA treatment. Each tumor was moderately or well differentiated and demonstrated no angiolymphatic invasion.

Following transplantation, steroid doses were tapered to 15 mg/d by postoperative day seven. Azathioprine was discontinued on postoperative day seven and maintenance immunosuppression with tacrolimus was begun. His first allograft biopsy three weeks after transplant revealed only mild ischemia-reperfusion changes and no evidence of rejection or recurrent HCV infection. The tacrolimus dose was adjusted to achieve a trough of 10-15 $\mu\text{g/L}$ for the first three months after transplant. After three months, transaminases were elevated and a liver biopsy done at this time revealed recurrent HCV with grade 1 (0-4) inflammation and steatosis without fibrosis (stage 0, 0-4). Prednisone dose was tapered and subsequently discontinued. Tacrolimus doses were further decreased to achieve a target trough level of 6-10 $\mu\text{g/L}$. A one-year liver biopsy, however, demonstrated progression to fibrosis (stage 2, 0-4). Transaminases remained mildly elevated and new hyperbilirubinemia (total bilirubin 29.1 $\mu\text{mol/L}$) was also noted. Quantitative HCV RNA testing revealed high-level viremia with viral tiers of 5.8 GU/L. HCV treatment with pegylated interferon and ribavirin was begun. After a 15 wk course of interferon-based anti-viral therapy, he had

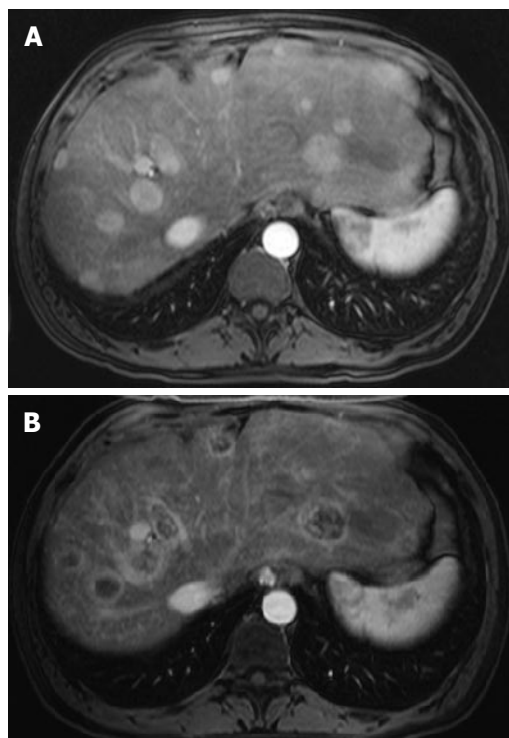


Figure 1 Pre-treatment magnetic resonance imaging of the liver demonstrating multifocal recurrent tumor within liver allograft (A) and paired post-treatment image showing central necrosis of lesions after treatment with yttrium-90 microspheres (B).

only a transient decrease in transaminases and bilirubin with persistently high HCV RNA levels. HCV treatment was discontinued.

HCC surveillance with serial imaging and AFP levels remained negative for the first 18 mo following transplantation. An elevated AFP level of 16 $\mu\text{g/L}$ was noted at 22 mo post transplant. A CT scan of the liver revealed a 3 cm nodule in segment VI not seen on the imaging five months earlier. Recurrent HCC was confirmed by needle biopsy, bone scan and chest CT were negative for distant metastatic disease. The patient was offered a segmental resection of the tumor and underwent laparotomy. No extra-hepatic tumor was identified. Intraoperative ultrasound revealed two additional 1 cm lesions in segments III and IV. These and the initial segment VI lesion were segmentally resected. The patient's post-operative course was uncomplicated. Post-operative AFP level decreased to 7 $\mu\text{g/L}$. One month after surgery, maintenance immunotherapy was switched from tacrolimus monotherapy to sirolimus monotherapy with target trough levels of 5-8 $\mu\text{g/L}$ and adjuvant chemotherapy with systemic doxorubicin (200 mg/m² over 20 wk) was begun. Chemotherapy was complicated by the development of diabetes mellitus and anemia. AFP level increased to 820 $\mu\text{g/L}$ despite chemotherapy. A CT scan done 3 mo into his course of doxorubicin showed vague hyper-enhancement in segment VIII and magnetic resonance imaging (MRI) scan of the liver obtained at the completion of treatment revealed uniformly enhancing, multifocal nodules consistent with HCC in the remaining liver allograft with the largest nodule measuring 3.8 cm (Figure 1A).

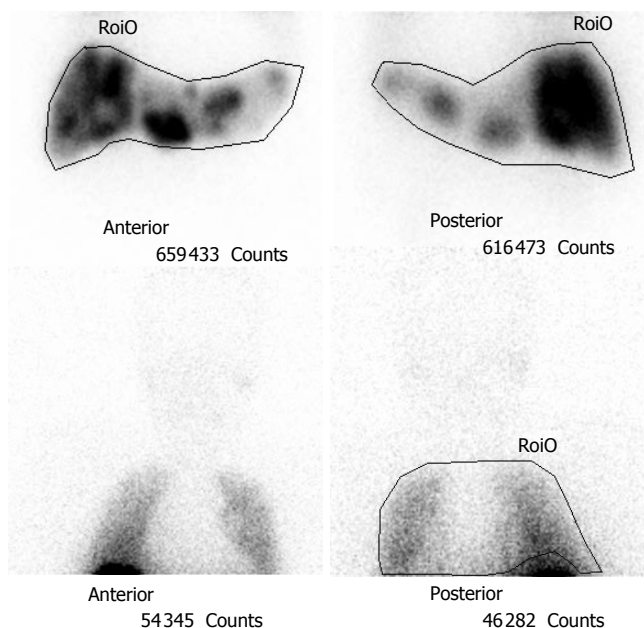


Figure 2 Pre-treatment screening by injection of macroaggregated albumin to determine shunt fraction to lungs.

After presentation at a multidisciplinary tumor board it was decided to proceed with a trial of hepatic intra-arterial radiotherapy with yttrium-90 microspheres (SIR-Spheres). Pre-procedure evaluation with hepatic arteriography was notable for the absence of collateral vessels to the allograft, given that the liver was supplied by the donor vessels and the native gastroduodenal artery was ligated at the time of transplantation. Injection of macroaggregated albumin to determine the degree of hepato-pulmonary shunting revealed a radioactive shunt fraction of 7.3% (Figure 2). CT volumetry of the affected liver allowed calculation of a targeted tumor volume of 120.6 cm³. He underwent yttrium-90 intra-hepatic arterial injection 32 mo after liver transplantation. The patient received a dose of 1.5 GBq of isotope. The procedure was uncomplicated and he was discharged to home six hours after the procedure was completed. The patient experienced only minimal symptoms related to the treatment, including mild right upper quadrant abdominal pain and intermittent nausea. Follow-up MRI of the liver was done two months post-treatment. Pre- and post treatment MRI images (Figure 1A and B) demonstrated loss of uniform arterial enhancement and development of peripheral enhancement consistent with tumor necrosis and treatment response. The number of lesions remained stable. Post-procedure AFP levels decreased to 20 µg/L at 2 mo following the procedure. Liver function, both clinical and biochemical, was unaffected by the treatment.

DISCUSSION

Following liver transplantation, recurrence of HCC that is isolated to the liver occurs with a frequency of around 3%-5% with the current liver transplant candidate selection criteria^[2-4]. Most recurrences occur within the first two years of transplantation and the two-year mortality after recurrence is high, approaching 75%^[4]. Recurrent

HCC may be heralded by impairment in graft function and elevation of AFP levels. Treatment measures usually include reduction of immunosuppression and local tumor therapy. Hepatic recurrence is multifocal in 60% of cases and tumor grade is T3 or greater in up to 90% of cases^[3]. As a result, impaired hepatic function along with multifocal disease often limits the potential for surgical resection. Local ablative techniques such as RFA, cryoablation or percutaneous ethanol ablation require repeated administrations and also have limited efficacy when there is extensive or diffuse tumor burden. TACE has evolved as a useful tool for treatment of HCC recurrence because it can be utilized for treatment of bilateral, multifocal lesions with efficacy and limited repeated administration^[6]. However, treatment requires infusion of chemotherapy and embolization of the selected hepatic artery. Systemic and local toxicity as a result of chemotherapy and hepatic ischemia is not rare^[6].

This is the first known report of hepatic intra-arterial brachytherapy with yttrium 90 microsphere (SIR-Spheres) for the treatment of HCC in a liver transplant recipient. Intrahepatic yttrium 90 microsphere (SIR-Spheres) has been used for the treatment of unresectable HCC and metastatic hepatic tumors^[7,8]. Yttrium-90 isotopes incorporated into a resin sphere slightly larger than the size of the capillary in the liver arterial system are delivered intra-arterially. Yttrium 90 emits beta-radiation and the permanently implanted spheres deliver a therapeutic dose radiation with an effective mean range of about 2.5 mm. The half-life of the isotope is 64 h with 95% of the dose given in the first 11 d. Higher doses of radiation are able to be delivered selectively than with external beam radiation therapy (EBRT). In addition, large segments of liver are not rendered ischemic because embolization is not a feature of the therapy. Therefore, there is less toxicity to functioning parenchyma than seen with EBRT or TACE.

Therapy is typically administered in one cycle, with a range of one-to-three cycles. Suitable candidates for treatment include patients with unilobar or bilobar disease and preserved liver function with bilirubin < 34.2 µmol/L^[7]. Contrast enhanced CT imaging of the liver serves to determine tumor volume and allow calculation of radiation dose requirement. In order to minimize treatment-related toxicity, hepatic arteriography is necessary to determine arterial anatomy and administer 99 mTc-macro-aggregated albumin to calculate the shunt fraction delivered to the lungs. In most patients, arteries that arise from the hepatic arteries and supply the stomach or intestines must be embolized to prevent radiation necrosis. If the hepatic arterial supply cannot be isolated, then the patient is generally considered not to be a candidate for therapy. Because of the isolated arterial supply of the liver allograft in our patient, this was not an issue. Pulmonary shunt fraction greater than 15% on 99 mTc-macro-aggregated albumin scan predisposes to radiation pneumonitis and precludes safe treatment with yttrium 90.

The median radiation dose for a treatment is 134 Gy which is delivered in a range of 50-150 Gy^[7]. The dose of yttrium 90 equates to 5-10 GBq or 2-4 million microspheres. Treatment is typically provided on an outpatient basis and does not require patient isolation

because yttrium 90 is a beta emitter. Local response to treatment is demonstrated by decrease in size and tumor vascularity. Thirty-eight percent of patients experience partial response demonstrated by a decrease in tumor size^[7]. In 65% of patients, response is demonstrated by substantial decrease in tumor vascularity^[7]. Monitoring of tumor markers such as AFP may also be of benefit in determining response to therapy^[8]. Impact on survival varies according to the severity of underlying hepatic disease but the median survival time after yttrium 90 microsphere (Theraspheres, MDS Nordion, Ottawa, Canada) treatment was 23 mo in one study of 65 patients with unresectable HCC^[7]. This represents an increase in survival of 2.6-4.7 times that of historic control. One multicenter review of treated patients reported that death resulting from therapy-induced liver failure is responsible for half of the treatment-related deaths and occurs in 5% of all patients^[9]. All of these liver failure deaths attributed to the yttrium 90 therapy are occurred in patients felt to be at high risk for the procedure. When compared to TACE, quality of life measures is higher at 12 mo^[10]. Toxicities of yttrium-90 include worsening ascites, lower extremity edema, nausea, vomiting, fever, abdominal pain, hyperbilirubinemia and lymphopenia^[7,8]. Mild adverse effects include nausea, vomiting fatigue, abdominal pain, fever^[7,8]. Serious adverse effects include tumor lysis syndrome, transient elevations in liver enzymes, gastritis, gastric or duodenal perforation, cholecystitis, and radiation induced pneumonitis^[7,8].

A recent multicenter review of low risk patients treated with yttrium 90 microspheres reported that liver toxicities including elevation of bilirubin and serum transaminase levels and ascites occur after treatment in 42% of patients, fifty percent of these adverse events are thought to be related to treatment, and toxicities are resolved in 78% of patients on short term follow-up^[11]. Development of toxicity is associated with pretreatment bilirubin > 17.2 $\mu\text{mol/L}$ and radiation dose > 137 Gy. There are no occurrences of radiation-induced liver disease or death related to treatment^[11]. Another multicenter review on the use of intrahepatic yttrium 90 microspheres has reported a three-month post-treatment mortality of 18%. Ten percent of deaths are thought to be related to treatment and occur in association with at least one or more of the following risk factors: presence of infiltrative tumor, tumor bulk > 50%, albumin < 30 g/L, bilirubin \geq 34.2 $\mu\text{mol/L}$, AST or ALT > five times the upper limit of normal, and a lung radiation dose of > 30 Gy^[9]. The results of these and other reports suggest that treatment in carefully selected patients is safe and efficacious.

Hepatic intra-arterial yttrium-90 microsphere treatment was safely and effectively used in this patient with

recurrent multifocal HCC after liver transplantation. Clearly, further prospective investigation is needed to determine whether this therapy can be applied with similar safety and efficacy to other patients with recurrent HCC after liver transplantation. The availability of this treatment modality in the post-transplant setting may help to prolong patient and graft survival in those recipients unfortunate enough to develop recurrent HCC who are not amenable to other treatment options.

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Successful management of hepatic artery pseudoaneurysm complicating chronic pancreatitis by stenting

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Abstract

A 41-year old alcoholic male with a history of chronic pancreatitis was admitted for nausea, vomiting and weight loss. Angiogram was performed and demonstrated an aneurysmal sac with a narrow neck originating from the inferior aspect of the distal portion of the proper hepatic artery. The origin of the pseudoaneurysm was covered with a 5 mm × 2.5 cm Viabahn cover stent (Gore). A repeat angiogram showed some leak and a second stent (6 mm × 2.3 cm) was deployed and overlapped with the first stent by 3 mm. Contrast was injected and a repeat angiogram demonstrated complete exclusion of the aneurysm. A repeat computerized axial tomography (CAT) scan of the abdomen after 24 h showed successful stenting. The patient had an uneventful post-operative course.

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Key words: Hepatic artery; Aneurysm; Stents

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INTRODUCTION

Hepatic artery pseudoaneurysm (HAP) is a serious complication of acute or chronic surgical injury to the hepatic artery. It is also seen following blunt and penetrating abdominal injury as well as in patients with chronic pancreatitis and after orthotopic liver transplantation^[1]. Transcatheter embolization has been considered the treatment of choice but we report a case of HAP successfully treated by stenting of the pseudoaneurysm.

CASE REPORT

A 41-year old male with a history of chronic alcohol consumption and chronic recurrent pancreatitis presented to the emergency department with abdominal pain, nausea and weight loss. Physical examination revealed remarkable ascites. No bruit was present. Serum upper gastrointestinal (GI) endoscopy revealed grade 1 esophageal varices. Abdominal ultrasonography (USG) demonstrated dilated pancreatic ducts. Abdominal MRI/MRA showed a lesion in the porta hepatis measuring 5 cm × 5 cm. Contrast-enhanced CAT scan of the abdomen showed a 6 cm × 6 cm pseudoaneurysm of the proper hepatic artery (Figure 1). Stenting of the pseudoaneurysm was done. The technique could selectively catheterize the proper hepatic artery by using a 5 French RC-1 catheter (Angiodynamic-Queensbury-NY). Angiogram was performed and demonstrated an aneurysmal sac with a narrow neck originating from the inferior aspect of the distal portion of the proper hepatic artery (Figure 2). The origin of the pseudoaneurysm was covered with a 5 mm × 2.5 cm Viabahn cover stent (Gore). A repeat angiogram showed some leak and a second stent (6 mm × 2.3 cm) was deployed and overlapped with the first stent by 3 mm. Contrast was injected and a repeat angiogram demonstrated complete exclusion of the aneurysm (Figure 3). A repeat computerized axial tomography (CAT) scan of the abdomen after 24 h showed successful stenting (Figure 4). The patient had an uneventful post-operative course.

DISCUSSION

Hepatic artery pseudoaneurysms are rare. However, rupture is common and occurs in 76% of patients. The mortality of patients requiring operative intervention is 75%^[1]. Pseudoaneurysm formation in pancreatitis is thought to occur because of autodigestion of pancreatic enzymes, especially elastase, liberated due to pancreatitis. Although angiography remains the gold standard for detection of pancreatitis, USG and CAT scan are often diagnostic and may provide an early mechanism for identifying patients with pseudoaneurysms occurring consequently to pancreatitis^[2]. Otherwise diagnosis is usually made late in the course of disease when severe or even fatal hemorrhage has occurred. Transcatheter embolization is the standard of care.

Literature search revealed one case report of a 60-year old female with HAP as a complication of surgical treatment of Klatskin tumor treated with a

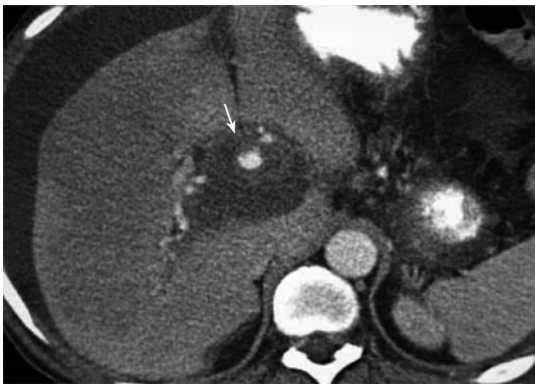


Figure 1 Contrast-enhanced CAT of the abdomen showing a 6 cm × 6 cm pseudoaneurysm of the proper hepatic artery.

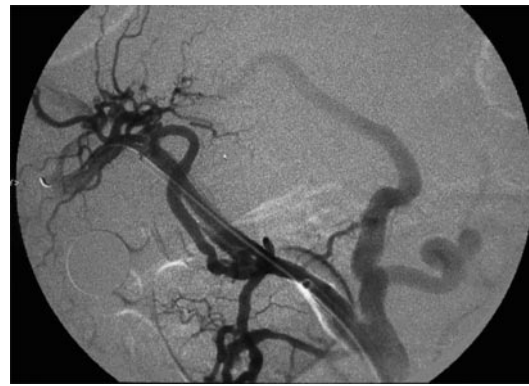


Figure 3 Repeat angiogram demonstrating complete exclusion of the aneurysm.

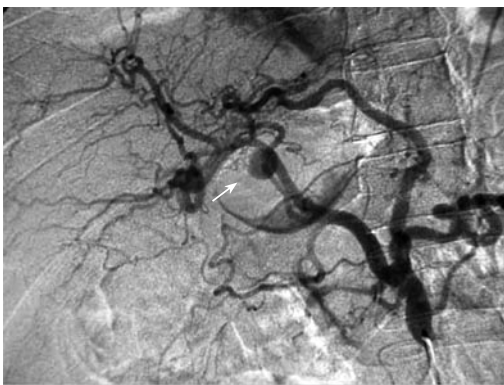


Figure 2 Angiogram showing an aneurysmal sac with a narrow neck originating from the inferior aspect of the distal portion of the proper hepatic artery.



Figure 4 A repeat CAT scan of the abdomen after 24 h showing successful stenting.

coronary stent-graft via transfemoral approach^[3]. Two other cases have been reported, one being stenting of HAP secondary to liver transplantation following unsuccessful coil embolization^[4] and the other being stenting of HAP secondary to therapeutic management of a choledochol cholangiocarcinoma, following failed arterial embolization^[5]. In our case HAP developed as a complication of chronic alcoholic pancreatitis with symptoms of abdominal pain. Early diagnosis with CAT scan followed by stenting resulted in complete occlusion of the pseudoaneurysm.

The long-term data regarding the use of this or other stent-grafts in the visceral vessels are extremely limited, and consequently we cannot recommend stent-grafts as the primary treatment option for visceral artery pseudoaneurysms. However, we expect that, with continued follow-up of cases such as these, further refinements in stent-graft design, greater availability, and increased operator experience, these devices will become an increasingly valuable therapeutic option. Early diagnosis and intervention with stenting, which is a reasonable

alternative to embolization, can result in excellent long-term outcome.

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Obstructive jaundice due to hepatobiliary cystadenoma or cystadenocarcinoma

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Abstract

Hepatobiliary cystadenomas (HBC) and cystadenocarcinomas are rare cystic lesions. Most patients with these lesions are asymptomatic, but presentation with obstructive jaundice may occur. The first patient presented with intermittent colicky pain and recurrent obstructive jaundice. Imaging studies revealed a polypoid lesion in the left hepatic duct. The second patient had recurrent jaundice and cholangitis. Endoscopic retrograde cholangiopancreatography (ERCP) showed a cystic lesion at the confluence of the hepatic duct. In the third patient with intermittent jaundice and cholangitis, cholangioscopy revealed a papillomatous structure protruding into the left bile duct system. In the fourth patient with obstructive jaundice, CT-scan showed slight dilatation of the intrahepatic bile ducts and dilatation of the common bile duct of 3 cm. ERCP showed filling of a cystic lesion. All patients underwent partial liver resection, revealing HBC in the specimen. In the fifth patient presenting with obstructive jaundice, ultrasound examination showed a hyperechoic cystic lesion centrally in the liver. The resection specimen revealed a hepatobiliary cystadenocarcinoma. HBC and cystadenocarcinoma may give rise to obstructive jaundice. Evaluation with cross-sectional imaging techniques is useful. ERCP is a useful tool to differentiate extraductal from intraductal obstruction.

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Key words: Liver; Hepatobiliary cystadenoma; Cystadenocarcinoma; Obstructive jaundice; Endoscopic retrograde cholangiopancreatography

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INTRODUCTION

Hepatobiliary cystadenomas (HBC) are rare neoplasms of the liver or extrahepatic bile ducts, accounting for less than 5% of all the cystadenomas found in the liver. These lesions are mainly seen in middle-aged females^[1,2] and can show malignant degeneration to hepatobiliary cystadenocarcinoma. Most of these patients are asymptomatic, because these lesions usually are incidental findings on abdominal diagnostic imaging for evaluation of other complaints. If there are presenting symptoms, these are right upper quadrant pain or discomfort. Obstructive jaundice only rarely occurs as a presenting symptom^[3]. We describe here the diagnostic evaluation, surgical management and pathological characteristics of four patients with HBC and one patient with hepatobiliary cystadenocarcinoma who presented with obstructive jaundice.

CASE REPORT

Case 1

A 50-year old woman underwent laparoscopic cholecystectomy because of symptomatic cholelithiasis 3 years prior to presentation. She was admitted for intermittent colicky pain and recurrent biliary obstruction. Physical examination showed no abnormalities. Routine liver function tests revealed elevated plasma levels of alkaline phosphatase (AP) of 118 U/L (normal range, 40-120 U/L) and gamma-glutamyltransferase (GGT) of 260 U/L (normal, < 40 U/L). Serum bilirubin level was normal. Endoscopic retrograde cholangiopancreatography (ERCP) demonstrated a polypoid lesion inside the left hepatic duct. Helical computed tomography (CT) of the liver revealed a cystic lesion measuring 10 cm in diameter located in the left lobe of the liver with protrusion into the left hepatic duct. Calcifications were present in the wall of the lesion and septations were visible inside the cyst (Figure 1A). ERCP showed the image of an extrinsic mass proximal to the polypoid lesion, creating a compression on the intrahepatic ducts of the left biliary system (Figure 1B). These findings suggested that the episodes of colicky pain and cholestasis were caused by a cystic lesion with a polypoid protrusion into the left bile duct, giving rise to intermittent obstruction. Distinction between hepatobiliary cystadenoma and cystadenocarcinoma could not be made on the basis of imaging studies. The patient underwent left hemihepatectomy and

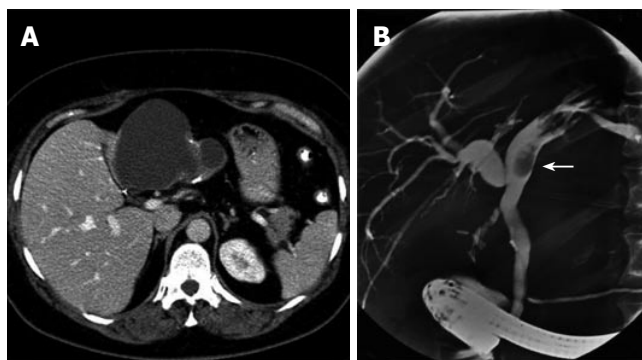


Figure 1 Abdominal CT-scan showing a large cystic mass in the left liver lobe with internal septations and calcifications in the cyst wall (A) and ERCP showing a polypoid lesion in the left hepatic duct (arrow) in case 1 (B).

the histopathological diagnosis of hepatobiliary mucinous cystadenoma was made. The postoperative course was uneventful and the complaints resolved after operation. The patient died 56 mo after operation because of other reasons not related to the initial disease.

Case 2

A 46-year old woman underwent cholecystectomy with common bile duct exploration because of gallstones 6 years prior to presentation. During cholecystectomy, fenestration of a presumed simple intraluminal cyst at the confluence of the hepatic duct was performed. Microscopic examination of the cyst wall showed biliary epithelium without mesenchymal stroma. She had recurrent jaundice and cholangitis. Initial investigations revealed elevated levels of serum bilirubin level of 79 $\mu\text{mol/L}$ (normal, < 17 $\mu\text{mol/L}$), AP of 311 U/L (normal range, 40-120 U/L), GGT of 448 U/L (normal, < 40 U/L), AST of 96 U/L and ALT of 142 U/L (normal, < 45 U/L). ERCP showed filling of a cystic lesion at the confluence of the hepatic duct. Brush cytology revealed no malignancy. A plastic stent was placed for decompression of the biliary system. Abdominal ultrasonography (US) and CT-scan revealed multiple cystic lesions involving segment 4 of the liver with dilatation of the left central and peripheral intrahepatic bile ducts and the right central intrahepatic bile ducts. At laparotomy, multiple cysts in segment 4 of the liver were found with a dominant cystic lesion of 2.5 cm located in the area of hepatic hilum. Hilar resection in combination with left hemihepatectomy was performed. Microscopically, the cyst wall was composed of cylindrical epithelium. The features of the subepithelial stroma were typical for the diagnosis hepatobiliary mucinous cystadenoma. The complaints resolved after operation. The patient was still alive 103 mo after operation.

Case 3

A 40-year old woman underwent laparoscopic cholecystectomy for symptomatic cholecystolithiasis 4 mo ago. She had intermittent complaints of jaundice and cholangitis. Liver function tests showed slightly elevated AP levels of 103 U/L and GGT level of 44 U/L. The serum bilirubin and alanine transaminase levels were normal. ERCP revealed a filling defect in the left hepatic duct (Figure 2A).

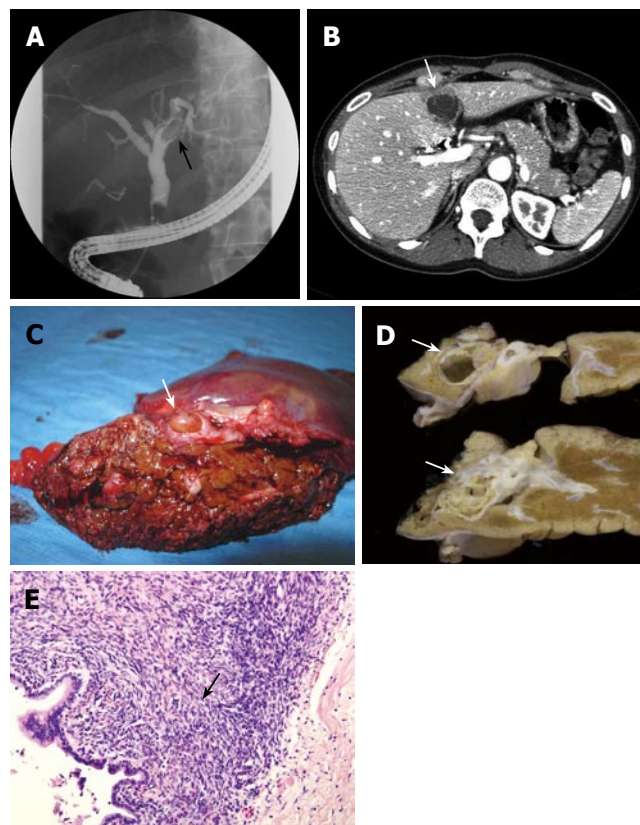


Figure 2 ERCP showing a filling defect due to an intraluminal lesion (arrow) in the left hepatic duct (A), CT-scan showing a cystic lesion with internal septations measuring 3.6 cm located in segment 4 (arrow) (B), specimen after left hemihepatectomy showing macroscopic features of a large lesion (arrow) inside the left bile duct filling up the entire lumen (C), macroscopic cut sections of a multicystic lesion (arrows) encapsulated by a thick fibrous capsule arising from the left hepatic duct (D), microscopical features showing columnar mucinous epithelium with underlying dense-cellular stroma resembling ovarian stroma (arrow) (HE X 200) (E) in case 3.

A stent was placed for decompression of the biliary system. The second ERCP at our hospital suggested that the filling defect was caused by an intraductal tissue mass. Subsequent cholangioscopy showed a papillomatous structure protruding into the left bile duct system from the bile duct wall. CT scan showed a cystic lesion with septations located in segment 4 of the liver. It was unclear if there was a connection between the lesion and the left hepatic duct. The lesion was 3.6 cm in diameter (Figure 2B). The patient underwent a left hemihepatectomy. Macroscopically, a tumour with smooth surface was seen protruding inside the bile duct and filling up the entire lumen (Figure 2C). Cut sections revealed a multicystic lesion encapsulated by a thick fibrous capsule arising from the left hepatic duct (Figure 2D). The cystic lesion was surrounded by stroma containing spindle-shaped cells, resembling ovarian stroma (Figure 2E). The diagnosis of hepatobiliary mucinous cystadenoma was made. No signs of malignancy were seen. The patient remained alive 19 mo after operation.

Case 4

A 43-year old woman with an unremarkable previous medical history presented with progressive obstructive jaundice three weeks ago. Abdominal US showed dilatation of in-

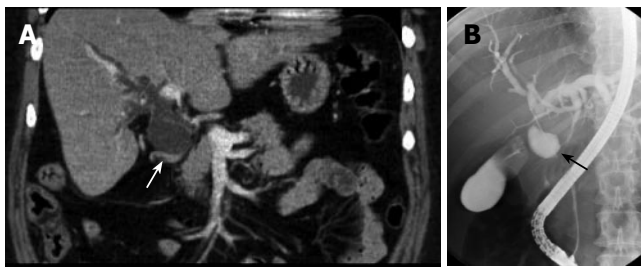


Figure 3 Abdominal coronal CT-scan showing dilated intrahepatic bile ducts and common bile duct (arrow) (A), ERCP showing filling of a cystic lesion (arrow) connected to the common bile duct, initially diagnosed as a duplicate gallbladder or choledochal cyst (B) in case 4.

tra- and extra-hepatic bile ducts and a collapsed gallbladder without a clear cause. ERCP showed a normal pancreatic duct but the common bile duct could not be visualized. CT showed slight dilatation of the intrahepatic bile ducts and dilatation of part of the common bile duct with a diameter of 3 cm (Figure 3A). Subsequent MRCP showed the same images as obtained with CT. ERCP showed filling of a cystic lesion connected to the common bile duct (Figure 3B). A diagnosis of a duplicate gallbladder or choledochal cyst at the level of the common hepatic duct was considered. A cystic lesion was detected during laparotomy and a decision was made to resect the common bile duct and the gallbladder. Continuity of the bile duct was restored using a hepaticojejunostomy. Histopathological examination of the resection specimen revealed a multilocular hepatobiliary cystadenoma with necrotizing inflammation. The patient was still alive 46 mo after operation.

Case 5

A 39-year old woman presented with obstructive jaundice, nausea and weight loss which began three months before presentation. Liver function tests showed elevated bilirubin levels of 128 $\mu\text{mol/L}$, AP of 1053 U/L, GGT of 500 U/L, AST of 172 U/L and ALT of 142 U/L. The alpha-fetoprotein level was normal. Because of failure of ERCP, percutaneous transhepatic drainage (PTD) was performed for biliary decompression via the right intrahepatic bile ducts (Figure 4A). Complete obstruction at the level of the proximal bile duct was seen. US showed a well defined hyperechogenic cystic lesion of 4.4 cm, centrally in the liver. A solid calcification of 1 cm was situated inside the lesion. CT showed a cystic lesion with irregularly thickened wall, in conjunction with dilated intrahepatic bile ducts (Figure 4B). Preoperative differential diagnosis of hepatobiliary cystadenoma, cystadenocarcinoma or choledochal cyst was made. The patient underwent hilar resection combined with extended right hemihepatectomy and biliary reconstruction using a hepaticojejunostomy. The diagnosis of hepatobiliary cystadenocarcinoma was made microscopically. The lesion was completely resected with microscopically free margins. The patient was still alive 59 mo after operation. Follow-up imaging showed no signs of local or distant tumor recurrence.

DISCUSSION

HBC are rare cystic neoplasms that may occur in the liver

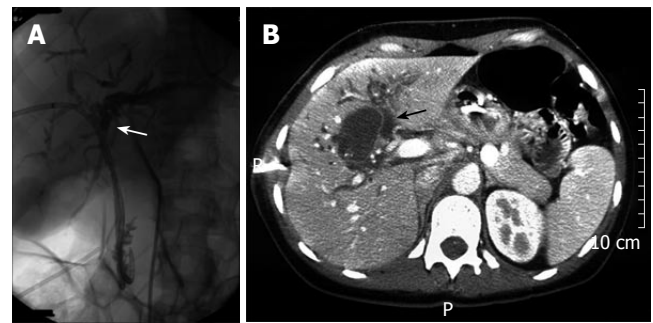


Figure 4 Percutaneous transhepatic drainage (PTD) showing complete obstruction at level of the proximal bile duct (A), abdominal CT showing a cystic lesion with irregularly thickened wall in conjunction with dilated intrahepatic bile ducts (arrow) (B) in case 5.

or in the extrahepatic biliary system. It is estimated that these cystic liver neoplasms comprise 5% of all cystic liver lesions^[1]. Only a minority of patients with HBC develop symptoms, the most commonly reported symptoms are right abdominal pain, abdominal swelling, anorexia or nausea. Rarely, patients present with colicky pain or jaundice caused by obstruction of the biliary system. The differential diagnosis of HBC without symptoms includes simple cysts, choledochal cysts, hepatobiliary cystadenocarcinoma, hydatid cysts, abscess or haematoma. Obstructive jaundice is usually a presenting symptom of choledocholithiasis or malignant cholangiocarcinoma.

In the past 14 years, 13 patients have been diagnosed with HBC or cystadenocarcinoma in our institution. Five of these patients (38.5%) presented with biliary obstruction, which is usually caused by external biliary compression or by internal obstruction due to a mass inside the bile duct. Mucus hypersecretion in case of HBC or cystadenocarcinoma communicating with the bile ducts may also give rise to obstruction symptoms^[4]. Three patients in this series presented with intermittent obstruction jaundice and two patients had continuous jaundice. In contrast, continuous biliary obstruction usually is the presenting symptom of patients with hilar cholangiocarcinoma.

Typically elevated cholestatic parameters in the blood are secondary to obstruction or compression of the biliary system. Some authors have suggested that serum CA-19-9 levels can be used for diagnosis or as a parameter of tumor activity during follow-up after resection^[5,6]. CA-19-9, a serum marker normally synthesized by normal pancreatic and biliary ductal epithelium, is elevated both in benign biliary lesions and in malignant pancreatic carcinomas^[7]. These serum markers, however, may be elevated in the presence of cholestasis and are therefore less reliable in the diagnosis of bile duct lesions. On imaging studies, distinction between HBC and simple cysts can be made by the presence of septations and irregularly thickened cyst walls with or without calcifications^[8]. The most accurate imaging methods for detecting cystadenomas are abdominal ultrasound and CT-scan^[9].

ERCP is useful, apart from establishing the diagnosis, in stenting the bile duct and decompressing the biliary system as was done in our patients. It can also help to differentiate between extraductal and intraductal obstructions.

However, it still remains difficult to differentiate between cystadenoma and cystadenocarcinoma by imaging methods^[10]. In patients with cystic lesion at the hepatic hilum, diagnosis of choledochal cyst should be considered as well^[11,12]. Liver hydatid disease, caused by *Echinococcus* granulosis, is usually asymptomatic and should also be considered as rupture of a hydatid cyst into the biliary tract, a well know complication, which also may give rise to colicky pain, cholangitis and obstructive jaundice. In these patients, ERCP is useful in detecting communication between the cystic lesion and the biliary system^[13].

The use of percutaneous biopsy for preoperative diagnosis has no additional value considering the fact that it rarely produces a definitive diagnosis. There is also the additional risk of peritoneal dissemination in case of malignancy. Histopathological examination is required for definitive diagnosis. Microscopically, the linings of HBC are composed of a biliary type, mucus-secreting cuboidal or columnar epithelium. The underlying stroma shows presence of ovarian stroma in 85%-90% of HBC^[14]. Distinction with simple liver cysts is made among other features, on the basis of the presence of subepithelial ovarian stroma in HBC. Simple liver cysts are composed of an outer layer of fibrous tissue and an inner lining of single columnar or cuboidal epithelium.

In the past, different treatment strategies such as partial resection, percutaneous aspiration or application of sclerosant agents inside the lesions have been applied to HBC. Patients treated with these techniques have shown high recurrence rates when compared to those who have undergone radical partial liver resection^[15]. Another reason for resection is the possibility of malignant degeneration, although the precise risk remains unknown. In our series, cystadenocarcinoma was only seen in 2 of the 13 patients (15.4%) with HBC, which is comparable with the incidence in literature ranging from 5% to 25%^[3,16]. The mean age of patients with cystadenocarcinomas has been reported to be approximately 17 years which is older than that of those without malignant degeneration^[14]. The risk of malignant degeneration is also supported by the presence of benign epithelium in the wall of most cystadenocarcinomas^[11]. Therefore, the treatment of choice should be radical surgical resection. The 5-year survival rate for cystadenocarcinomas after surgical resection ranges from 25% to 100%^[17,18]. We observed no recurrence of cystadenocarcinomas during a mean follow-up of 56 mo (range 19-103 mo) by abdominal ultrasound or CT.

In conclusion, hepatobiliary cystadenoma or cystadenocarcinoma should be considered in patients with obstructive jaundice in the presence of a cystic liver lesion. ERCP and cross-sectional imaging techniques have a great value for establishing the diagnosis.

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Benign multicystic peritoneal mesothelioma: A case report and review of the literature

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Abstract

Benign multicystic peritoneal mesothelioma (BMPM) is a rare tumor that occurs mainly in women in their reproductive age. The pathogenesis of BMPM is unclear and a controversy regarding its neoplastic and reactive nature exists.

The biological behavior of BMPM is characterized by its slowly progressive process and high rate of recurrence after surgical resection. In addition this lesion does not present a strong tendency to transform into malignancy. Today approximately 130 cases have been reported.

We here report a 62-year-old woman who had diffuse abdominal pain, nausea and vomiting. Physical examination revealed a painful mass in her upper abdomen. She reported a mild dehydration, but the vital signs were normal. Peristaltic rushes, gurgles and high-pitched tinkles were audible. Upright plain abdominal film revealed small bowel loops with air-fluid levels. She was diagnosed having an incarcerated incisional hernia that resulted in intestinal obstruction. The patient underwent surgery during which a cystic mass of the right ovary measuring 6 cm x 5 cm x 4 cm, four small cysts of the small bowel (1 cm in diameter) and a cyst at the retroperitoneum measuring 11 cm x 10 cm x 3 cm were found. Complete resection of the lesion was performed. The patient had an uneventful recovery and had no recurrence two years after surgery.

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Key words: Acute abdomen; Ovary; Peritoneum; Benign multicystic mesothelioma; Adenomatoid tumor

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INTRODUCTION

Benign multicystic peritoneal mesothelioma (BMPM), also known as multilocular peritoneal inclusion cysts, is an uncommon lesion arising from the peritoneal mesothelium that covers the serous cavity. This lesion occurs most frequently in women during their reproductive years^[1-3] and is associated with a history of previous abdominal surgery^[4], endometriosis^[5,6] or pelvic inflammatory disease^[4]. However, there are reports concerning men^[7-9] or children^[10-11], as well as rare extra-abdominal cases^[12-14]. To date, approximately 130 cases have been reported^[1,6,8,15-18]. While the origin of the disease is known, the pathogenesis and pathological differential diagnosis remain unclear and controversial. We here report a case of BMPM admitted to our department with a review of the literature.

CASE REPORT

A 62-year-old woman was referred to the Surgical Outpatient Department due to diffuse abdominal pain, nausea and vomiting for 24 h. On physical examination a painful mass in the upper part of a median subumbilical incision was palpated. She reported a history of hysterectomy five years earlier. A mild dehydration was noted, but the vital signs were normal. Peristaltic rushes, gurgles and high-pitched tinkles were audible. An upright plain abdominal film revealed small bowel loops with air-fluid levels. She was diagnosed having an incarcerated incisional hernia that resulted in intestinal obstruction. The patient underwent an emergency surgery during which a cystic mass of the right ovary measuring 6 cm x 5 cm x 4 cm, four small cysts of the small bowel (1 cm in diameter) and a cyst at the retroperitoneum measuring 11 cm x 10 cm x 3 cm were revealed. Complete resection of the lesion was performed. The pathology report was benign multicystic mesothelioma of the ovary, bowel and peritoneum (Figure 1). The patient had an uneventful recovery and was closely followed-up by US and CT. She remained free of symptoms and had no recurrence two years after surgery.

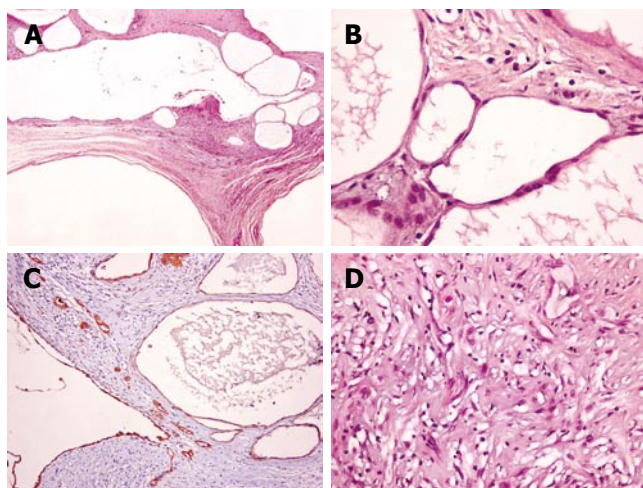


Figure 1 Histological and immunohistochemical findings in benign multicystic mesothelioma (A-C) and in ovary adenomatoid tumor (D) within collagenous stroma (A: HE x 20; B: HE x 400; C: AE1/AE3 x 100; D: HE x 400).

DISCUSSION

BMPM was first described in 1979 by Menemeyer and Smith^[19]. Since then approximately 130 cases have been reported^[20] and the information regarding BMPM is derived from a small number of patients from one institution or from isolated case reports. There are larger series reported from pathologist consultation files with patients from different institutions with incomplete clinical information and lack of long-time follow-up data^[11,17]. Therefore, this disease is classified as an exceedingly rare medical entity, which challenges its origin, pathogenesis, diagnosis and therapy.

Origin

BMPM, a localized tumor arising from the epithelial and mesenchymal elements of the mesothelial cells, does not metastasize. It has a strong predilection for the surface of the pelvic viscera. When the tumor is found in the peritoneal cavity, lesions are found intimately attached to serosal surfaces of the intestine and omentum or in the retroperitoneal space, spleen and liver^[21].

Pathogenesis

The pathogenesis of BMPM is a controversial entity. Some authors believe that the lesion is neoplastic, while others favor a reactive process^[1,15-17,22]. The close relationship with inflammation, a history of prior surgery, endometriosis or uterine leiomyoma suggests that BMPM is probably a peculiar peritoneal reaction to chronic irritation stimuli, with mesothelial cell entrapment, reactive proliferation and cystic formation. Microscopic examination of the lesion reveals an inflammatory component in many cases. The close association of BMPM with familial Mediterranean fever characterized by periodic fever and peritonitis reinforces this assumption^[23].

Other authors have proposed a neoplastic origin based on a slow but progressive growth of the untreated lesions, a marked tendency to recur after surgical resection, a low

incidence of previous abdominal infection and a high disease-related mortality^[11,15]. Malignant transformation of BMPM, an unusual occurrence, indicating a neoplastic nature underscoring the necessity of long-term follow-up has also been reported^[20].

BMPM is rarely associated with adenomatoid tumor, another benign mesothelial lesion with its neoplastic and hyperplastic pathogenesis still argued^[24]. Tumors with mixed histological features of multicystic mesothelioma and adenomatoid tumor have also been reported^[24,25]. These facts indicate that a histogenetic relationship has led some authors to suggest that BMPM represents possibly a borderline lesion between an adenomatoid tumor and a malignant mesothelioma.

Pathological differential diagnosis

Pathological differential diagnosis includes a number of benign and malignant lesions that present as cystic or multicystic abdominal masses. Benign lesions include cystic lymphangioma (cystic hygroma)^[8,15], cystic forms of endosalpingiosis^[26,27], endometriosis^[28], mullerian cysts involving the retroperitoneum^[29], cystic adenomatoid tumors^[30] and cystic mesonephric duct remnants^[1]. Malignant lesions include malignant mesothelioma^[17] and serous tumors involving the peritoneum^[17]. Among the benign lesions, the most important differential diagnosis is BMPM from cystic lymphangioma and adenomatoid tumor. Cystic lymphangioma is restricted to the mesentery, omentum, mesocolon and retroperitoneum but rarely reported in the ovary. On gross examination the cystic component is often chylous and microscopic examination reveals bounds of smooth muscle and aggregates of lymphoid tissue. The cystic spaces are lined by a single layer of flattened endothelial cells which are immunoreactive to vascular markers (CD31, CD34, factor VIII, and VEGFR3). Cystic adenomatoid tumors are easily confused with BMPM on macroscopic and histological examination. However, the cystic component is usually accompanied with a recognizable solid component. Occasionally short papillae lined by mesothelial cells are seen. There are cases of tumors with mixed features of both adenomatoid tumor and BMPM^[24,25], indicating that the two lesions are probably pathogenetically related. Cystic forms of endosalpingiosis differ from BMPM by the presence of a tubal type epithelium that may include peg cells, ciliated cells and/or secretory type cells. Blunt papillae and psammoma bodies may also present. The so-called "florid cystic endosalpingiosis" with multicystic involvement of either uterine or extrauterine sites appears to represent the extreme examples of this process^[26,27]. Endometrioid cysts typically containing dark chocolate-brown materials are composed histologically of endometrial stroma lined by endometrial-type epithelium. Commonly there is evidence of old or recent hemorrhage. Mullerian cyst is another benign condition that may be confused with BMPM. Nevertheless, as its name implies, it is composed of mullerian-type serous or mucinous epithelium containing smooth muscle fibers in the wall. The malignant conditions that mimic BMPM can be easily differentiated on the basis of malignant features including

cellular atypia, increased mitotic count, abnormal mitoses and destructive infiltration of the underlying stroma.

Diagnosis

In most cases, BMPM remains silent in abdominal cavity and may invade several underlying organs, and more rarely the retroperitoneum^[31]. Sometimes BMPM is manifested as acute abdomen. In the case described here the first sign of the lesion was revealed only during surgery, which was performed on an emergency basis. In fact, most patients are diagnosed incidentally during examination or laparotomies for other reasons. The available modern imaging techniques, such as ultrasonography, computerized tomography and magnetic resonance imaging, can demonstrate the lesion. However a differential diagnosis is difficult to be made from other cystic neoplastic or inflammatory lesions arising from these anatomical areas^[32-34]. From a clinical point of view, the differential diagnosis of BMPM from cystic tumors of ovaries is most important, since BMPM may be treated by local excision with preservation of the ovaries. Preoperative fine-needle aspiration biopsy of cystic lesions may help the differential diagnosis^[35-37]. The diagnosis can be confirmed by electron microscopy and immunohistochemistry^[38]. Benign cystic neoplasm with which BMPM is most likely confused is cystic lymphangioma, which occurs more commonly in males and may develop in children as well^[17], but rarely recurs^[8].

Therapy

Surgery is the only effective treatment for BMPM. Complete removal of the cystic lesion if possible, remains the mainstay of treatment and the only hope to avoid local recurrence. Aggressive surgical approaches including cytoreductive surgery with peritonectomy are recommended^[39]. However, some investigators who support more conservative methods particularly claim that treatment options and timing of surgery should be made individually based on the benign morphologic pattern of the lesion^[40]. The laparoscopic approach has also been attempted^[41,42]. Recurrences occur more frequently in women and can be shown by CT guided cystic aspiration^[43], and treated by hormonal therapy with anti-oestrogens^[44] and gonadotrophin-releasing analogues^[45], hyperthermic intraperitoneal chemotherapy^[46,47], and sclerotherapy with tetracycline^[48]. In any case the degree of success following these procedures varies. Adjuvant chemotherapy and radiotherapy are not indicated because these tumors have a prevailing benign character.

In conclusion, BMPM is a rare benign cystic tumor. Although its recurrence is high after surgical resection, it does not present a tendency to transform into malignancy. Two cases of malignant transformation^[20,49] out of the approximately reported 130 cases cannot establish the incidence of this change, even if its neoplastic nature is supported. Finally, a prolonged systematic follow-up of these patients, perhaps for life, is required since the lesion usually reappears and further resection or other therapy may be indicated. Moreover, this strategy may lead the investigators to have a definite aspect concerning the biological behavior of the disease.

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Activation of c-Yes in hepatocellular carcinoma: A preliminary study

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TO THE EDITOR

Hepatocellular carcinoma (HCC) is thought to develop through a multistep process^[1]. A long history of viral

hepatitis or prolonged exposure to environmental toxins predisposes liver cells to mutations of the genes critical in the control of hepatocyte growth. In fact, both activation of cellular oncogenes and inactivation of tumor-suppressor genes are involved in the development of HCC. Activation of oncogenes by hepatitis virus integration has been shown in the woodchuck animal model^[2], although the significance of this finding in human hepatocarcinogenesis is still under investigation. Tyrosine kinases, though a minor class of cellular protein, represent a major class of oncogenes. These tyrosine kinases are classified into two major groups^[3,4]. The first is receptor-type protein tyrosine kinases, the second is non-receptor type tyrosine kinases. The main representatives of the latter group are non-receptor-linked and membrane-associated Src family tyrosine kinases. At least nine Src-related tyrosine kinases have been identified thus far, including c-Yes, c-Src, c-Lck, c-Fyn, c-Hck, c-Lyn, c-Blk, c-Fgr and c-Yrk protooncogene products. The cellular oncogene c-Yes, a member of the Src family, encodes a 62-kilodalton, cytoplasmic and membrane-associated protein-tyrosine kinase^[5]. c-Yes expression and its kinase activity have been shown to be increased in colorectal cancer^[6,7], melanoma^[8] and metastatic liver cancer^[9]. However, the activation of c-Yes in human HCC has not yet been investigated at all. In the present study, we determined the activity of c-Yes both in the normal liver (NL) tissues and in chronic hepatitis (CH), tumorous (T) and adjacent nontumorous (N) cirrhotic liver tissues. This is the first report on the activity of c-Yes in various liver diseases including HCC.

Tissue samples, including the tumorous and surrounding nontumorous cirrhotic tissues, were obtained during surgery from 9 patients with HCC (7 males and 2 females; mean age, 68.2 ± 4.4 years; range, 59-73 years). All the patients were positive for hepatitis C virus (HCV), as determined by the reverse-transcriptase polymerase chain reaction method (Amplicor, Roche Diagnostics Ltd). The number of patients with well-, moderately- and poorly-differentiated HCC was 2, 5 and 2, respectively. The fibrosis stage of the surrounding liver tissues was assessed as cirrhosis in all the HCC cases. Liver tissue samples from three patients with HCV-induced CH were obtained by liver biopsy. During surgery, two normal liver tissue samples were also obtained from patients with liver metastases of colon cancer. The serum samples of these patients with NL were negative for HCV and hepatitis B virus (HBV).

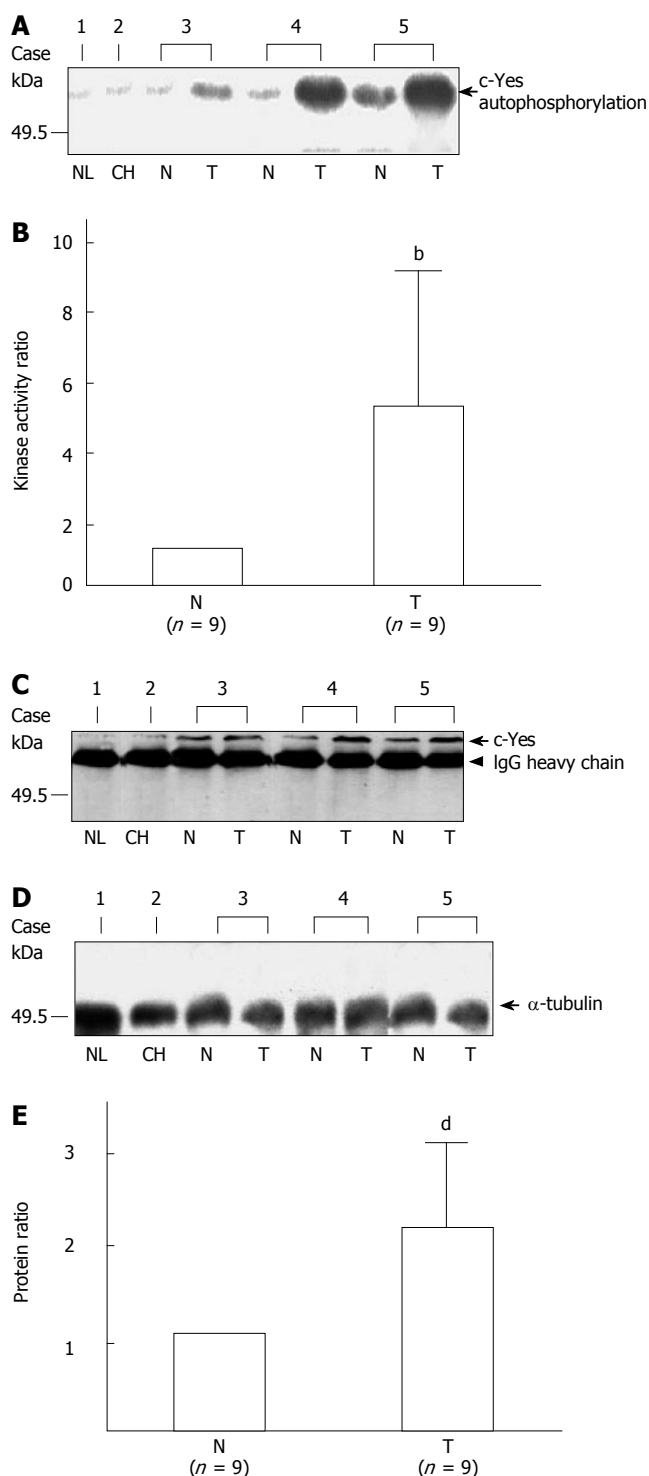


Figure 1 Activities and levels of c-Yes kinase in different liver tissues. **A:** Activity of c-Yes in normal liver (NL), chronic hepatitis (CH), nontumorous cirrhotic (N) and tumorous (T) tissues. The arrow indicates the band corresponding to c-Yes autophosphorylation; **B:** Relative levels of total c-Yes activity (mean \pm SE, Student's *t*-test, $^bP < 0.001$); **C:** Levels of c-Yes in the NL, CH, N, and T portions of HCC. The arrow and arrowhead indicate the bands corresponding to c-Yes protein and the heavy-chain of the c-Yes antibody, respectively; **D:** Level of α -tubulin in lysate containing 100 μ g of cellular protein used in the immunoprecipitation (arrow); **E:** Relative levels of c-Yes protein in T and N tissues (mean \pm SE, Student's *t*-test, $^dP < 0.01$).

To determine the protein kinase activity of c-Yes in the NL, CH, N and T portions of HCC, we prepared lysates containing 100 μ g of cellular protein of tissue samples, precipitated the protein with a monoclonal antibody specif-

ic for c-Yes [MAb (3H9), Wako Pure Chemical Co, Tokyo], and measured the autophosphorylation of c-Yes using an *in vitro* protein kinase assay described previously^[6,8]. Activities of c-Yes in the representative results of NL (patient 1), CH (patient 2), N and T tissues (patients 3-5), respectively, are shown in Figure 1A. When the kinase level of N tissues was used as a reference level ($n = 1$), c-Yes activity ratio in the T tissues ($n = 9$) was 5.0 ± 3.7 times higher than that in the N tissues, as measured by autophosphorylation (Student's *t*-test, $P < 0.001$) (Figure 1B). Patients 3-5 were classified as well-differentiated, moderately-differentiated and poorly-differentiated HCC, respectively. In addition, the c-Yes activity in all NL and CH tissues used in this study was very low as measured by autophosphorylation of c-Yes. Thus, c-Yes activity was notably high in human liver tissues with malignancy. In addition, our results showed that although the kinase activity of c-Yes was very low in NL and CH, it was already activated in liver cirrhosis (non-tumorous cirrhotic tissues). Because the kinase activity of c-Yes was already activated at the preneoplastic stage (cirrhosis), *i.e.*, before the development of HCC, these data suggest that HCV-induced liver cirrhosis is a precancerous condition.

Protein levels of c-Yes were determined by Western blot analysis. Immunoprecipitates used for the protein kinase assays were also applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to nitrocellulose membranes. As shown in Figure 1C, two proteins were detected in each lane. The upper protein was c-Yes, and the lower one was the heavy chain of the c-Yes monoclonal antibody. Although the level of c-Yes was very low in NL and CH, it was elevated in N and T tissues of HCC (Figure 1C). As an internal control, the amount of α -tubulin in lysate containing 100 μ g of cellular protein used in the immunoprecipitation was almost the same in each lane (Figure 1D). When the protein level of N tissues was used as a reference level ($n = 1$), the level of c-Yes protein in T tissues ($n = 9$) was 2.1 ± 0.84 times higher than that in the surrounding N tissues from the same patients (Student's *t*-test, $^dP < 0.01$) (Figure 1E). The ratio (T tissue *vs* surrounding N tissue) of the protein level of c-Yes was smaller than that of the kinase activity. These data suggest that the high c-Yes kinase activity in the HCC is probably caused not only by an increase of c-Yes protein but also by an increase of the enzyme activity.

In conclusion, activation of the protooncogene product c-Yes may play a significant role in the malignant transformation of hepatocytes. The suppression of c-Yes kinase activity may offer a novel strategy for overcoming the development and invasion of HCC. Further studies are necessary to investigate such processes.

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LETTERS TO THE EDITOR

Unilateral leg edema in a cirrhotic patient with tense ascites

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TO THE EDITOR

A 61 year old man with cirrhosis and hepatocellular carcinoma developed on the background of chronic hepatitis B was admitted because of acute and gradually intensified right thigh pain and swelling, which, within a few hours, was expanded to his right foot. The patient, due to ascites refractory to diuretics, was almost weekly subjected to large volume paracentesis of ascitic fluid for the last six months; meanwhile he has developed a left inguinal, a right femoral and an umbilical hernia. It should be noted that 48 h before admission, the patient was referred to the emergency room because of dyspnoea and 5 L of ascitic fluid were removed.

Physical examination was notable of tense ascites and his right lower extremity was profoundly more edematous (thigh, calf and foot) compared to his left leg (Figure 1), without signs of inflammation. Inguinal lymph nodes were not enlarged, Homan's sign was absent, and peripheral arteries were palpable. There was also a left inguinal hernia and an umbilical hernia containing fluid in the sac, while

there was no edema in the scrotum.

The patient was initially subjected to duplex ultrasonography of his right leg, which turned negative for deep venous thrombosis. Subsequently, a CT scan of the right thigh and abdomen was performed, revealing an enormous fluid collection within the peritoneal cavity (Figure 2A) and a notable fluid collection within the lateral aspect of the right thigh (Figure 2C). The fluid in the right thigh exhibited approximately the same density with ascitic fluid (Figure 2A and C). No masses were detected in the pelvis.



Figure 1 Significant edema of the right leg without signs of inflammation in our cirrhotic patient with tense ascites.

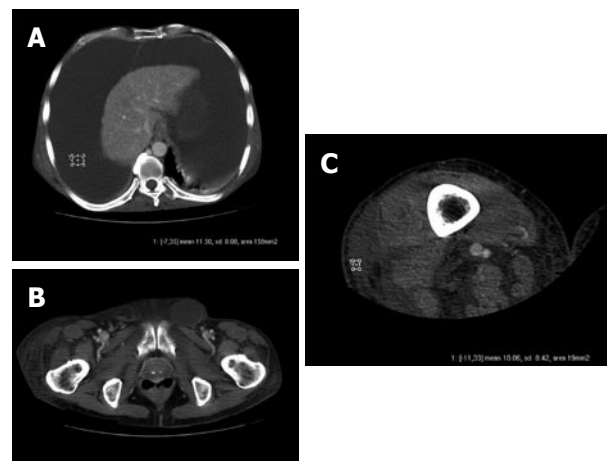


Figure 2 A: Enormous fluid collection within the peritoneal cavity. The fluid density was measured to be 11.5 Hounsfield Units; B: No masses were detected in the pelvis, whereas there was fluid collection into the left inguinal canal and no evidence of fluid collection into the right inguinal canal; C: Fluid collection within the lateral aspect of the right thigh with a density of 10.06 Hounsfield Units.

In addition, fluid was also seen in the left inguinal canal, but not in the scrotum, while no hernia was seen in the right femoral ring (Figure 2B). What could be the etiology

of the patient's right lower extremity edema?

According to the diagnostic evaluation performed, the possibility of deep venous thrombosis of the right leg was practically excluded based on negative duplex, increased international normalized ratio (INR) (2.2) and low platelet count (70 000/mm³) that our patient exhibited in the text of his cirrhosis. Also any mechanical reasons that could induce unilateral venous or lymphatic stasis were ruled out. However, according to the CT scan findings, it seemed that the patient's lower extremity edema was caused by accumulation of ascitic fluid, which somehow passed from the peritoneal cavity to the right thigh. Interestingly, the known right femoral hernia was detected neither by physical examination, nor by CT scan, tempting us to speculate that drainage of fluid contained within the femoral hernial sac to the thigh might have occurred.

Asking in more detail the patient's history we were informed that in his prior admission to the emergency room (48 h ago), he was subjected to right femoral vein puncture for blood sampling. A potential mechanism of ascitic fluid accumulation in the right thigh could be the injury of the right femoral hernial sac during femoral vein

puncture. The femoral hernial sac and its content pass through the femoral ring and are generally localized between the external femoral vein and Gimbernat's lacunar ligament medially. These hernias, having penetrated Scarpa's triangle, through stretching of the cribrate lamina, are visible subcutaneously in the thigh. Reasonably, perforation of the femoral hernial sac would permit the drainage of ascitic fluid to the subcutaneous tissue of the right thigh, forced by the high intraabdominal pressure, whereas the scrotum would remain unaffected (unlike the case of an inguinal hernia perforation). The patient was subjected to repeated large volume paracentesis and along with ascites improvement his right leg edema was significantly decreased.

This case presents a rare complication of femoral vein puncture in a patient with tense ascites, which raised diagnostic dilemmas and led to expensive diagnostic evaluation. Clinicians handling cirrhotic patients should be aware of this potential complication and perform femoral vein or artery puncture with caution or ideally under scan control in case of coexistence of ascites and femoral or inguinal hernias.

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LETTERS TO THE EDITOR

Diagnostic dilemma between intestinal Behçet disease and inflammatory bowel disease with pyoderma gangrenosum

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TO THE EDITOR

I have read with great interest the very recent article titled "Intestinal Behçet's disease with pyoderma gangrenosum: A case report" of Nakamura T *et al* that was published in your journal. The authors stated that they presented a very rare case of intestinal Behçet's disease with pyoderma gangrenosum in a 16-year old patient. However, I would like to make some important contributions and suggestions to the presented case and have a few questions to ask the authors.

First, the exact diagnosis of Behçet disease in a single case depends first on the recognition of a characteristic set of sufficient symptoms and/or signs to allow the physician to diagnose with various levels of certainty from "complete or definitive" Behçet disease to "suspected or possible" Behçet disease^[1] based on the clinical sign constellation in Japanese Behçet Disease Research Committee Criteria, the preferred diagnostic criteria before 1989^[2]. According to the criteria, such a "complete" or "definitive" diagnosis of Behçet disease needs either at least three major criteria or two major criteria (one of which is ocular disease) or two major criteria associated with 2 minor criteria. This means that the correct diagnosis of Behçet disease can be established only on

the basis of aforementioned strict rules that need the complete fulfillment of the criteria with careful differential diagnosis from other etiologies. Therefore, the authors' statement that "she was diagnosed with intestinal Behçet's disease by the presence of cutaneous pathergy together with two major criteria (oral and genital aphthoses) and one minor criterion (gastrointestinal manifestations) in 1984" cannot be accepted as the presented case does not fulfill the requirements of "Japanese Behçet Disease Research Committee Criteria", the preferred diagnostic criteria in that period, in which cutaneous pathergy is not included in the major or minor criteria. Although oral aphthous and genital ulcerations with a positive pathergy test of the presented case meet the diagnostic criteria of International Study Group^[3], published in 1990, however, diagnosis and surgical intervention of the presented case with "incomplete diagnosis" were made in 1989. In other words, the case can be accepted as "suspected" Behçet disease according to the Japanese criteria, provided that other etiologies for these manifestations have been strictly ruled out including inflammatory bowel disease (IBD). Therefore, the authors should first clarify this diagnostic confusion.

Second, the presented case was stated to have colitis involving the entire colon demonstrated by colonoscopy. However, typical intestinal involvement in Behçet disease is characterized mainly by changes in the small intestine or ulcerative lesions at the terminal ileum or cecum, resulting in various digestive symptoms^[4]. I think the differential diagnosis was not performed strictly enough in this single case as the aforementioned symptoms are seen in IBD, namely Crohn's disease and ulcerative colitis that need careful evaluation. Although the resected specimen demonstrated severe inflammation with neutrophil accumulation, this is a general finding in inflammatory diseases and intestinal Behçet disease is often indistinguishable from IBD by histological evaluation. Therefore, such a general finding cannot be used as the histological hallmark of intestinal Behçet disease as its diagnosis is based on the presence of deep colonic ulcerations frequently situated in healthy mucosa along with the presence of an adjacent non-specific inflammatory infiltrate affecting the entire colonic wall that is characterized by leukocytoclastic vasculitis and perivasculitis of the arteries and veins with signs of fibrinoid necrosis. Moreover, there is not any knowledge in the presented article about the presence or absence of granulomatous or non-granulomatous changes or caseation with or without confluent (diffuse) or skipping

(segmental) lesions (*i.e.*, mural thickening) that strongly need strict differential diagnosis of intestinal Behçet disease from IBD. Indeed, ulcerative colon, for instance, is restricted to the colon, and exhibits proximal extension over time with full thickness involvement in case of toxic megacolon^[5]. Therefore, nothing in the article can exclude these questions in that single case that seems to need clarification.

Third, the authors stated that the patient received 20 mg oral prednisolone treatment for a month, though cutaneous and intestinal lesions of the girl were poorly controlled. However, to our knowledge, corticosteroids (CSs) alone are not used for the treatment^[1] and management^[6] of Behçet disease at least in the used dose, and no study has found that CSs alone are effective on any symptom or sign as well as on any etiological factors including cutaneous and intestinal lesions that are accused for the pathogenesis of Behçet disease^[7-13]. Moreover, CSs are of little value for the maintenance of remission. Indeed, the results of Mat C *et al*^[14] are important and demonstrate that CSs alone for about a six- month duration are not effective even on oromucocutaneous symptoms despite the well-known underlying vasculitic pathology of the condition. Because most of the treatments have been shown to work in Behçet disease or in IBD, the used dose of CSs seems not sufficient for such a severe intestinal involvement in the present case and therefore, the patient should have been treated first with a higher dose of CSs (1-2 mg/kg prednisolone per day) for a short time with some other immunomodulating/immunosuppressive agents or their combinations with cyclophosphamide, chlorambucil, colchicine, dapsone, cyclosporine and especially azathioprine that could be used in that period for the management and treatment of complete or incomplete Behçet disease patients with or without intestinal findings, to induce rapid and durable remission of intestinal attacks before the decision of an invasive surgery (total colectomy) is made^[15-21]. In other words, whether the patient is supposed to suffer from Behçet disease or IBD, the indication for surgical treatment can be made upon failure of sufficient and appropriate medical treatment or based on the severe complications such as abscess, fistula formation and iatrogenic perforation. Although systemic CSs may be useful in the early stages of severe inflammatory attacks, they are of limited value for long-term management of serious involvements whereas sustained remission can be accomplished only by the immunomodulatory agents. For instance, azathioprine or chlorambucil associated with high dose CSs should have been tried initially for the girl to obtain possible remission with long-term steroid-sparing effects. Similarly, cyclophosphamide- and cyclosporine-CS combinations have also been used between 1971 and 1989 for various symptoms of Behçet disease, even in cases resistant to conventional therapy^[22-28]. Furthermore, Sanderson^[29] has evaluated chronic IBD in children including Crohn's disease, ulcerative colitis, indeterminate colitis and Behçet's colitis in the review in that period and stated that treatment with drugs (sulphasalazine, steroids, azathioprine) and elemental diet are helpful and concluded that the prognosis of chronic IBD in childhood is good. More importantly,

sulphasalazine with or without cyclosporine and CSs has successfully been used for the treatment of both intestinal Behçet disease and IBD^[30-32].

Fourth, the authors further stated that intractable ulceration of the left foot surprisingly disappeared postoperatively after total colectomy within two weeks with no relapse of pyoderma gangrenosum for 10 years afterwards, suggesting a close relationship between pyoderma gangrenosum and intestinal Behçet disease. I do not agree with the authors on this regard and I believe that if the patient had really had Behçet disease, pyoderma gangrenosum or any other kind of cutaneous manifestations might have developed in its due course as the disease is known to be most active during the second and third decades of life. Indeed, the authors further stated in their paper that pyoderma gangrenosum might respond to surgical resection of the associated diseases, such as ulcerative colitis and Crohn's disease (see discussion section, second paragraph), and then stated in the following sentences that pyoderma gangrenosum in the present case rapidly improved after total colectomy. I agree on this regard and fortunately, total colectomy results in cure of IBD with reasonable long-term benefit in many cases. Therefore, both the authors' statements in their paper and the literature again indicate that the girl had an IBD with or without Behçet disease.

Although aphthous punched-out ulcerations may be found in about one-tenth of Behçet disease patients, they occur most frequently in the terminal ileum and cecum^[33] and this finding alone is still not sufficient for the diagnosis of Behçet disease as the case should strictly fulfill the whole systemic diagnostic criteria as stated above and aphthae in the colon may be seen not only in Behçet disease, but also, for instance, in Crohn's disease^[34]. Moreover, a very recent study has demonstrated that none of the clinical parameters of a total of 162 consecutive adult patients with diagnosis of IBD consisting of Crohn's disease and ulcerative colitis fulfill the Behçet disease diagnostic criteria^[35]. In other words, Behçet disease was diagnosed in none of the patients even though they had various symptoms such as intestinal involvement, pyoderma gangrenosum, articular disease, skin lesions, and oral ulcer with or without HLA-B51 positivity. Furthermore, such case reports with identical and perplexing clinical presentations as compared to those of Nakamura *et al*, are still present in the literature, describing association of both diseases^[32,36,37].

In conclusion, although I agree that the gastrointestinal and systemic features of Behçet disease and IBD overlap to a considerable extent that need addressing both in terms of increasing our understanding of pathogenesis and improving therapy, they are generally viewed as two distinct diseases, and oral aphthous ulcerations, genital ulcerations, papulopustular lesions or pyoderma gangrenosum on the leg with or without pathergy test positivity are all encountered findings during the course of IBD. In addition, constellation of findings for the diagnosis of Behçet disease is applicable only in the absence of other clinical explanations such as IBD. Therefore, the absence of sufficient symptoms to diagnose this young girl as "complete" or "definitive" Behçet disease

along with the presence of active colitis and ulcerations involving the entire alimentary tract, mainly colon in that case, reaching up to the terminal ileum associated with a rapid disappearance of intractable foot ulceration after surgery with the lack of demonstrated thrombotic or leukocytoclastic vasculitis (typical hallmark of Behçet disease) on colonic histopathological examination in the presented case, strongly indicates a diagnosis of pyoderma gangrenosum associated with IBD characterized by Behçet disease-like clinical presentation^[38-40]. Consequently, I suggest that the authors of the present case perform HLA analysis in their patient. If it reveals HLA-B51 positivity, then the very unusual diagnosis of classified or unclassified IBD associated with Behçet disease can be made in that case. If it reveals HLA-B27 positivity, then the patient can be classified in that case as HLAB-27 positive IBD. Even if the patient is still to be accepted as having intestinal Behçet disease, it seems that the rules for the treatment of such an involvement have not been followed before the surgery.

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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
isvhl2006@mci-group.com
www.isvhl2006.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
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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
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European Society of Clinical Microbiology
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3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
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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

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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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- 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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Gastric atrophy, diagnosing and staging

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Abstract

H pylori is now accepted as the cause of gastritis and gastritis-associated diseases, such as duodenal ulcer, gastric ulcer, gastric carcinoma, and gastric MALT lymphoma. The natural history of *H pylori* gastritis includes inflammation progressing from the antrum into the adjacent corpus resulting in an atrophic front of advancing injury leading to a reduction in acid secretion and eventual loss of parietal cells and development of atrophy. Sub-typing intestinal metaplasia has no clinical value to the patient, the pathologist, or the endoscopist. The pattern, extent, and severity of atrophy, with or without intestinal metaplasia, is a far more important predictor than is intestinal metaplasia subtype. The challenge remains to identify a reliable marker that relates to pre-malignant potential.

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GASTRITIS DUE TO *H PYLORI* INFECTION

H pylori is now accepted as the cause of gastritis and gastritis-associated diseases, such as duodenal ulcer, gastric ulcer, gastric carcinoma, and gastric MALT lymphoma. Overall, two rules are clear: (1) the pattern of gastritis is the major determinant of disease outcome^[1,2], and (2) countries with a high prevalence of gastric cancer and gastric ulcer, such as Japan or Peru, have a low incidence of duodenal ulcer^[3]. Duodenal ulcer is typically associated with antral predominant gastritis, little or no atrophy and normal or increased acid secretion^[4-7]. Gastric ulcer and intestinal gastric cancer are typically associated with extensive gastritis, widespread intestinal metaplasia and

hypo- or achlorhydria^[3,4,8,9]. However, both rules can be broken^[9,10]: (1) wide spread intestinal cancer has been documented in the corpus of Korean duodenal ulcer patients, and (2) both diseases (duodenal ulcer and gastric cancer) are frequent diagnoses in dyspeptic Korean patients^[9,10]. One of the keys to this apparent paradox is a person's natural acid secretory status.

DUODENAL ULCER AND GASTRIC ULCER REPRESENT TWO ENDS OF ONE DISEASE "*H PYLORI* INFECTION"

Although *H pylori* are found throughout the stomach, in the early stages of disease, *H pylori*-associated inflammation is often mild, superficial, or even absent in the gastric corpus^[10,11]. The natural history of *H pylori* gastritis is for the inflammation to progress from the antrum into the adjacent corpus resulting in an atrophic front of advancing injury, leading to a reduction in acid secretion and eventually loss of parietal cells and development of atrophy^[10,12,13]. This progression is not inevitable. In the general population it progresses at a rate of 1%-2% per year^[12]. The rate of progression of gastritis differs among different countries, different regions of the same country and among different *H pylori*-related diseases^[14,15]. Overall, the incidence of gastric cancer is highest in countries and regions with a high incidence of early development of atrophic corpus gastritis^[12,16-19] (Figure 1). In contrast, in duodenal ulcer patients, gastritis tends to stay largely confined to the antrum and either does not advance, or spreads very slowly, to involve the gastric corpus^[12,20,21]. Atrophic pangastritis with hypochlorhydria is rare or develops sufficiently late in life that the risk of gastric cancer for the population of patients with duodenal ulcer remains low.

The rate of progression of *H pylori* gastritis progression depends on the acid milieu. Thus, *H pylori* corpus gastritis is accelerated in clinical scenarios associated with low acid secretion, such as chronic therapy with proton pump inhibitors, which are widely used in gastro-esophageal reflux disease^[8,22-37]. Omeprazole therapy is associated with a reduction in bacterial load, both in the antrum and in the corpus, and a tendency for antral histology to improve and corpus gastritis to either not change or worsen. With omeprazole therapy, not only does the corpus mucosa fail to show histologic improvement, but there is a significant progression of the inflammatory reaction deeper within the pit involving the proliferative zone^[38].

A person's natural acid secretory status thus appears to determine whether they will develop duodenal or gastric

ulcer disease^[39,40] with the acid secretory status appearing to affect both the distribution and severity of *H. pylori*-related gastritis. There is some evidence that some cases of duodenal ulcer disease may “burn out” and this has been postulated to be due to the extension of gastritis into the corpus, thus reducing acid secretion to the point where it is no longer possible to sustain an active duodenal ulcer^[41]. One possibility is that with continued inflammation, antral atrophy may lead to a sufficient destruction of gastrin producing cells^[42] to produce a fall in acid secretion^[43,44], which would allow the development of corpus gastritis. In most duodenal ulcer cases, gastritis extends slowly or not at all giving the impression of localization to the antrum^[45]. Thus, antral predominant gastritis may in some instances represent an earlier stage of atrophic pangastritis such that these patterns actually represent two ends of the spectrum of “*H. pylori* infection” rather than mutually exclusive diseases^[10,46,47] (Figure 1).

The rate of progression from gastritis to atrophy varies in different geographic regions related to other environmental factors. While diet is probably the most important factor that reduces acid secretion, other factors such as childhood infections may be very important^[10,14,48,49]. The rate of development and the proportion of the population with atrophic gastritis is a critical determinant for the risk of gastric cancer in that population^[14,15]. The apparent higher prevalence of concomitant duodenal ulcer and gastric cancer in Korea^[46] and the presence of atrophic gastritis with intestinal metaplasia in the corpus of Korean duodenal ulcer patients^[10] suggest that in Korea the rate of expansion of the atrophic front is more rapid than in patients in other geographic areas.

DIAGNOSING AND STAGING GASTRIC ATROPHY

This review only covers the histopathological diagnosis and staging of gastric atrophy; serologic measures are not addressed. The natural history of *H. pylori* gastritis is to go through a cascade of events that involves non-atrophic gastritis, atrophic gastritis, and finally dysplasia^[50-52]. Atrophy begins at the fundic- or B-boundary line (defined as a margin between the corpus, with complete fundic gland mucosa, and the antrum)^[18,53,54] as a sheet of pseudo-pyloric metaplasia with islands of intestinal metaplasia^[10,13,55] and shifts proximally such that the antrum appears to expand replacing fundic gland mucosa with advancing atrophic gastritis^[18,20,53,56]. Corpus atrophy progresses proximally to variably sized regions of the adjacent greater curve, proximal half of the lesser curve, and neighboring anterior and posterior walls of the corpus^[15,18,53,57]. We will address intestinal metaplasia and pseudo-pyloric metaplasia separately.

INTESTINAL METAPLASIA

Because the development of gastric carcinoma is a slow and unpredictable process, and intestinal metaplasia is an easily recognizable marker for atrophy, investigators have suggested that sub-typing intestinal metaplasia using high-

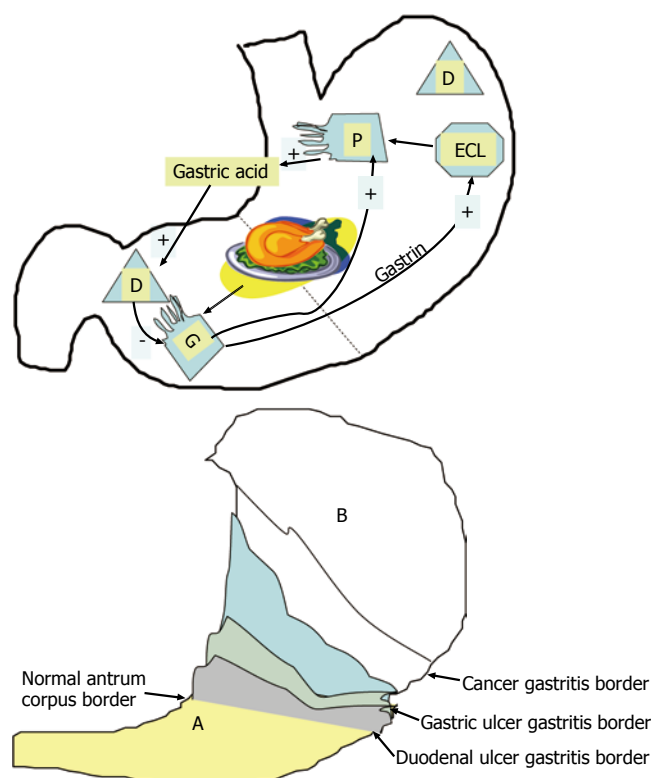


Figure 1 Duodenal ulcer and gastric ulcer represent two ends of one disease “*H. pylori* infection”. Unlike gastric ulcer patients, duodenal ulcer patients have a long lag period before developing gastric atrophy. Disease progression is dependent on *H. pylori* infection (cured/uncured) and other environmental factors such that in some countries DU would be considered protective against the development of gastric carcinoma.

iron diamine staining might identify subgroups of patients with different risk potential. Intestinal metaplasia sub-typed as III is often considered as a precursor lesion for the intestinal form of gastric cancer^[58-61]. In practice, areas of intestinal metaplasia (or a certain sub-type) are generally small and can easily be missed at follow-up^[62]. Sampling error is likely the critical factor responsible for the fact that an approximately equal number of studies have suggested that intestinal metaplasia regresses or does not regress after treatment of *H. pylori* infection^[62-70]. Prior studies suggesting an association of type III intestinal metaplasia with the development of gastric cancer^[59-61,71] did not take into account the higher prevalence of incomplete intestinal metaplasia (type III) in the gastric antrum^[13,72,73]. In addition, while type III intestinal metaplasia is present in all specimens with intestinal type gastric carcinoma, it can easily be missed in biopsy as it can be present in very small areas^[13].

A small percentage of cancer patients can show complete replacement of the antrum mucosa with intestinal metaplasia and have normal appearing oxyntic mucosa^[13]. It is unknown if these individuals had normal or reduced acid secretion. Continued inflammation with antral atrophy could possibly lead to sufficient destruction of gastrin producing cells^[45], which can result in a fall in acid secretion^[74,75]. Alternatively, contiguous sheets of intestinal metaplasia may be unstable epithelium especially upon exposure to carcinogens.

Pepsinogen I (PG I) normal corpus vs metaplasia (pseudopyloric metaplasia)

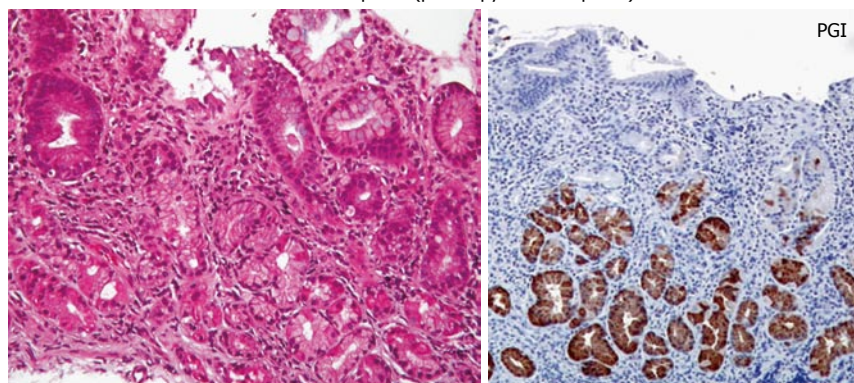


Figure 2 The diagnosis of pseudopyloric metaplasia can be facilitated by using pepsinogen I immunostain. Pepsinogen I (PG I) is localized in chief cells, mucous-neck cells and transitional mucous-neck/chief cells of the human fundic mucosa^[78] and is negative in antral gland cells.

Table 1 The diagnosis of pseudopyloric metaplasia can be facilitated by using pepsinogen I immunostain

	Pepsinogen I (PG I)	Pepsinogen II (PG II)
Chief cells	Positive	Positive
Mucous neck cells	Positive	Positive
Antral gland cells	Negative	Positive

Pepsinogen I (PG I) is localized in chief cells, mucous-neck cells and transitional mucous-neck/chief cells of the human fundic mucosa^[78]; it is not localized in antral gland cells. Pepsinogen II, on the other hand is localized in chief cells, mucous neck cells, and antral gland cells.

Overall, it is apparent that it is not currently possible to make recommendations or prognoses based on either a single or multiple biopsies showing sulphomucin in areas with intestinal metaplasia^[62,72,76,77]. All data suggest that the extent of mucosal atrophy within a region of the stomach may have a more important relation with the intestinal type of gastric cancer than the presence or type of intestinal metaplasia. While intestinal metaplasia is a form of atrophy that is easy for pathologists to recognize, it is also important to determine whether intestinal metaplasia is present as an isolated patch within non-atrophic mucosa or amidst an atrophic lawn^[10,13].

PSEUDO-PYLORIC METAPLASIA

The normal oxyntic mucosa has straight glands composed of tightly packed chief cells, parietal cells, endocrine cells, and mucus cells with a higher ratio of glands to foveola than the antrum. With continuous inflammation, and progressive atrophy, there is a progressive loss of parietal cells. Eventually, the oxyntic mucosa glandular compartment can resemble antral/pyloric glands on H&E exam (pseudopyloric metaplasia). The diagnosis of pseudopyloric metaplasia can be facilitated by using pepsinogen I immunostain (e.g. anti-pepsinogen I from Biogenesis Kingston, NH). Pepsinogen I (PG I) is localized in chief cells, mucous-neck cells and transitional mucous-neck/chief cells of the human fundic mucosa^[78]; it is not localized in antral gland cells (Figure 2). Pepsinogen II, on the other hand is localized in chief cells, mucous neck cells, and antral gland cells (Table 1). Pseudo-pyloric metaplasia is identified by the presence of mucosa that

is phenotypically antrum, stains positive for pepsinogen I, and is anatomically in a region where corpus would be expected^[13,55].

Pseudopyloric metaplasia has been described as early as 1959^[79] in benign gastric ulcers proximal to the normal border zone (antrum-corpus junction). In fact, prior to the rediscovery of *H. pylori*, a proximally advancing atrophic front with pseudopyloric metaplasia was considered part of the normal aging process^[18,56]. Following the rediscovery of *H. pylori*, a positive association has been demonstrated between the presence of mucous glands in corpus biopsies (pseudo-pyloric or mucous metaplasia) and the age of *H. pylori* infected patients. This association was more prevalent in Korea where gastric carcinoma is common^[10]. The pattern of atrophy in the form of pseudo-pyloric metaplasia is considered regenerative in nature^[80,81] and has been observed in experimental models^[82], as well as in gastric remnants following distal gastrectomy with gastroenteric anastomosis^[83]. In fact, routine screening for gastric cancer in asymptomatic patients with gastric remnants often reveals pseudo-pyloric metaplasia in oxyntic type mucosa.

STAGING ATROPHY

In staging corpus atrophy, it is important to remember four rules: (1) atrophy begins at the border line (antrum-corpus border); (2) atrophy replaces fundic gland mucosa with both pseudopyloric metaplasia and/or intestinal metaplasia^[13,53,84]; (3) the atrophic border extends proximally more rapidly up the lesser curve than the greater curvature such that locations high on the greater curvature are among the last to manifest atrophy^[13,53,79,84]; and (4) the presence of a higher density of mucosa mononuclear cells that infiltrate deep into the lamina propria is a predictor for the presence of gastric atrophy^[38,85].

In early stages of atrophic gastritis, observed in children^[55], the location of the antral-corpus border would be expected to be nearer to the normal anatomic border^[10]. As such, the identification of atrophy requires biopsies be taken close to the normal antrum corpus junction^[55]. In contrast, the atrophic front (atrophic border) is expected to be more proximal in patients in developing countries, in countries with a high incidence of gastric carcinoma (Figure 1), and within particular groups in developed

countries that have a higher incidence of gastric carcinoma, including the socially and economically disadvantaged^[86], with the atrophic border advancing more proximally with age^[10]. To note, the cardia is not only a high yield zone for *H pylori*^[11], but also both intestinal metaplasia and pseudo pyloric metaplasia have been identified in the cardia of children with early atrophic gastritis^[55].

The Sydney system and Updated Sydney system^[87] were primarily designed to provide standardization for reports of gastric biopsies. The Sydney system^[88] recommended a minimum of two biopsies from the respective gastric compartments to be taken from the anterior and posterior wall. In 1994, the Sydney system for the classification and grading of gastritis was updated. The recommendation was unchanged regarding the need for a minimum of two biopsies from the respective gastric compartments but the location was changed from the anterior and posterior walls to the greater and lesser curves of the stomach^[87]. In both instances the sites were chosen arbitrarily. Though the Sydney biopsy sites have proven to provide reliable identification of *H pylori* infection^[44,89,90], the sites recommended by the Sydney system can only identify corpus atrophy when it is extensive^[10,62,90,91]. The recommended biopsy sites for research studies designed to identify the presence, pattern, or changes in atrophic gastritis over time or following a therapeutic intervention must be carefully selected to ensure that they encompass the advancing atrophic border. The number and sites chosen will therefore depend on the average degree and severity of atrophic gastritis expected in the population^[13,55]. Use of a standardized reporting system, such as the updated Sydney system, is useful for biopsy specimens particularly as it promotes the use of a visual analog scale to score mucosal findings. For research we suggest the use of a 6 point scale^[92] as it provides finer gradation than the 4 point scale used for reporting clinical specimens^[87].

In summary, to increase our likelihood of identifying corpus atrophy, when present, special emphasis should be placed on: (1) targeting biopsy sites to encompass the likely sites of the advancing atrophic border and the cardia^[55], (2) consistently including intestinal metaplasia and pseudopyloric metaplasia in our evaluation of corpus biopsies for atrophy^[13,87], and lastly, (3) raising our suspicion for corpus atrophy in biopsies with a higher density of mucosa mononuclear cells that infiltrate deep into the lamina propria^[38,85].

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Non-surgical treatment of esophageal achalasia

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Abstract

Esophageal achalasia is an infrequent motility disorder characterized by a progressive stasis and dilation of the oesophagus; with subsequent risk of aspiration, weight loss, and malnutrition. Although the treatment of achalasia has been traditionally based on a surgical approach, especially with the introduction of laparoscopic techniques, there is still some space for a medical approach. The present article reviews the non-surgical therapeutic options for achalasia.

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Key words: Achalasia; Botulinum toxin; Pneumatic dilatation

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INTRODUCTION

Esophageal achalasia is a rare neuromuscular disorder characterized by degenerative changes of myenteric plexus leading to a selective loss of inhibitory nerve endings. The consequences of this damage are the irreversible loss of peristaltic contractions and the impaired relaxation of the lower esophageal sphincter (LES)^[1]. The ultimate cause of ganglion cell degeneration is unknown, but an association with class II HLA antigens^[2,3] and some virus infections^[4,5] has been described. An autoimmune pathogenesis in achalasia has been hypothesized due to the description of

antimyenteric neuron antibodies in a subset of patients^[6], although a genetic predisposition cannot be excluded^[7,8].

If untreated, in due course the disorder causes a progressive stasis and dilation of the oesophagus; with subsequent risk of aspiration, weight loss, and malnutrition. Because the etiology of achalasia remains obscure, the treatment is strictly palliative and is aimed at reducing the basal and residual LES pressure. Thus, esophageal emptying is allowed by gravity. The treatment of achalasia has traditionally relied on a surgical approach; the advent of minimally invasive surgery with a shorter hospital stay, reduced morbidity, and quicker return to daily activity, makes this option even more attractive^[9,10]. However, the high cost of this approach, the access to reference centers, the surgeon's learning curve, and some methodological debates, are still an issue.

In contrast, there is presently recent evidence that some medical strategies may be of benefit in many patients with this disorder^[11-13]. This review summarizes the current knowledge of non-surgical management of achalasia.

PHARMACOLOGIC TREATMENT

A number of pharmacological agents have been used to decrease the LES pressure. However, most acute and chronic studies were uncontrolled ones, and usually included only a small number of patients. Very few single- or double-blind, placebo-controlled trials are available. Overall, the clinical efficacy is often poorly described, even though several studies have shown that some compounds may be temporarily useful, while waiting for a more definitive therapeutic option. Table 1, Table 2, and Table 3, report the results of the most acute, chronic, and controlled studies, respectively.

The nitrates are claimed to be effective in achalasia; however, the relatively high frequency of side effects and the lack of controlled studies limits their clinical use^[14]. Therefore, the calcium channel blockers have been more frequently used. In particular, nifedipine has the wider published clinical and experimental evidence of efficacy. The therapeutic schedule suggests a sublingual administration of 10-20 mg, 15-30 min before meals. Its efficacy varies largely (between 50% and 90% in clinical trials), with side effects complained by up to 30% of patients. These include peripheral edema, headache and hypotension, and may wane over time. To date, however, nitrates and nifedipine could be recommended only in patients with an early stage disease, as a temporary measure before a more definitive option is selected.

Table 1 Acute drug studies in esophageal achalasia

Author	Drug	n	% of LES decrease
Wright (1961)	Butylschopolamine	3	Decreased
Von Weiser (1977)	Nifedipine	6	30
Becker (1981)	Verapamil	3	Unchanged
Cargill (1982)	Nifedipine	6	Decreased
Hongo (1982)	Nifedipine	8	45
Di Marino (1982)	Carbuterol	10	55
Nashrallah (1983)	Nifedipine	7	55
Becker (1983)	Verapamil	7	31
Traube (1984)	Nifedipine	20	30
Wong (1987)	Terbutaline	15	Decreased
	Aminophyllin		
Bassotti (1988)	Nitroglycerin	9	70
Guelrud (1992)	VIP	6	51
Marzio (1994)	Cimetropium	20	Decreased
Penagini (1994)	Loperamide	9	Decreased
Bortolotti (1994)	Isosorbide	9	49
	Nifedipine	7	65
Bortolotti (2000)	Sildenafil	7	50

Table 2 Chronic drug studies in esophageal achalasia

Author	Drug	n	% efficacy	Follow-up (mo)
Yon (1975)	Anticholinergics	7	14	12
Gelfond (1981)	Isosorbide	24	79	2-19
Silverstein (1982)	Diltiazem	8	50	6
Gelfond (1982)	Nifedipine	15	53	8-14
Traube (1983)	Nifedipine	14	65-80	6
Maksimak (1986)	Nifedipine	4	LES decrease	3-6
Garia (1987)	Nifedipine	20	LES decrease	4
Coccia (1992)	Nifedipine	14	77	21

Table 3 Controlled drug studies in esophageal achalasia (NA = not available)

Author	Drug	n	% efficacy	LES decrease	Follow-up (mo)
Lobis (1976)	Dicyclomine	10	NA	42%	Acute
Bortolotti (1981)	Nifedipine	20	90	40%	6-18
Nashrallah (1985)	Nifedipine	4	75	Unchanged	1
Traube (1989)	Nifedipine	10	NA	28%	1
Triadafilopoulos (1991)	Nifedipine	14	NA	Decreased	1
	Verapamil	14	NA	Decreased	1

Table 4 Effect of BoTx on esophageal achalasia (only studies with at least 30 patients are reported)

Authors	Toxin units	n	% LES decrease	Response 1 mo (%)	Response 6 mo (%)	Response 12 mo (%)
Pasricha, 1996	80	31	45	90	55	-
Fishman, 1996	100	60	-	88	-	46
Cuilliere, 1997	80	56	31	75	60	-
Annese, 1998	100	57	55	88	55	35
Prakash, 1999	80	42	-	90	64	41
Kolbasnik, 1999	100	30	28	77	37	29
Annese, 1999	100-250	78	42	86	61	-
Annese, 2000	Variable	118	34	82	53	-
D'Onofrio, 2002	100	37	-	84	-	67
Storr, 2002	100	40	-	-	68	-
Martinek, 2003	100-250	49	65	93	-	41
Zaninotto, 2004	100	40	-	-	66	34

BOTULINUM TOXIN TREATMENT

Botulinum neurotoxins (represented by seven serotypes, abbreviated BoTx A to G) cause a sustained inhibition of neurotransmitter release at cholinergic terminals. These toxins specifically bind the presynaptic membrane, and enter the cytosol of the nerve terminal where they cleave different proteins involved in neuroexocytosis. The botulinum toxin A, which is currently used for the treatment of various dystonic skeletal muscle disorders, cleaves the SNAP-25 molecule at the presynaptic membrane, thus blocking the acetylcholine release and causing a denervation atrophy. Its beneficial effects are currently being extended to a variety of diseases with increased cholinergic function^[15-17].

The above considerations prompted Pasricha and colleagues to evaluate, for the first time, the usefulness of intrasphincteric injection of BoTx in esophageal achalasia^[18,19]. The rationale was that the selective loss of the inhibitory nerves in achalasia upsets the excitatory (cholinergic) influences on LES. Thus, by blocking the release of acetylcholine, locally injected BoTx might reduce the LES pressure and improve the "passive" esophageal emptying. These authors developed a protocol

that has become, with slight modification mostly due to the different toxin dosages, the standard one currently used in almost all centers. The toxin is injected through a standard sclerotherapy needle during an upper endoscopy performed under conscious sedation, and 80 to 100 Units of BoTx A are injected in each quadrant of the LES at 4 to 8 sites in 1 or 0.5 mL aliquots. Following the first observation, several investigators have found a success rate of 70% to 100% in relieving symptoms in the short-term with a parallel decrease of LES pressure and improvement of esophageal emptying, although probably to a lesser extent than that obtained after pneumatic dilation.

Table 4 summarizes most of the largest studies published on BoTx in achalasia; after a single injection of toxin, almost 80% of patients report good to excellent relief of symptoms. More than half are still in remission at six months and one third at one year. This waning of efficacy was expected on the basis of neurological experience^[20]. The presynaptic nerve endings of skeletal muscles start to sprout new branches after 2-3 mo thus re-innervating the neuromuscular junction; for this reason, in a neurological setting, the injection of toxin is repeated every 3-4 mo. The effect of toxin on gastrointestinal smooth muscle and myenteric plexus has yet to be elucidated; nevertheless, the mean duration of a single injection of toxin in esophageal achalasia is 10-12 mo, with a wide variability ranging from three months up to three years. The reason for such variability is unknown,

but is probably related to another drawback of the toxin: the development of an autoimmune response with the production of antibodies that may in turn decrease its efficacy in some patients. This problem, however, could be solved with the use of different serotypes (i.e. BoTx C) which bind to different presynaptic receptors^[21].

In the attempt to optimize the dose and time of administration of toxin, two multicenter studies, coordinated by our centers, have been recently performed. The first trial^[22] demonstrated the relative similarity in the efficacy of 100 Units of Botox[®] (Allergan) with 250 Units of Dysport[®] (Ipsen, U.K.), the other commercially available toxin A in our country. Another study found the advantage of early repetition of the toxin with a second injection of 100 Units after one month^[23]. This schedule allowed a remission rate of 75% at one year compared with a 35% rate obtained with the same amount (200 Units) in a single injection. Interestingly, this schedule has been proven to be effective in older (or aging) (more than 75 years) achalasic patients in inducing symptom remission^[24].

Only a few studies are available on the long-term efficacy of BoTx. Pasricha *et al.*^[25] reported a 30% efficacy rate after a mean follow-up of more than two years, despite repeated injections. In our experience, however, 72% of patients were still in remission after a mean follow-up of 18 mo, provided that the toxin was repeated when symptoms relapsed^[26]. More recently, after a mean follow-up of 49 mo (range 6-100 mo), we found a 70% rate of good or excellent control of symptoms in a series of 149 patients (Annese V, *et al.*, unpublished data).

Overall, the endoscopic injection of toxin has been remarkably safe, and this is a common experience with this approach^[27]. In less than 10% of patients, a mild chest pain develops shortly after the procedure, but this usually does not require specific treatment. More importantly, in case of failure, the injection of toxin does not influence the functional results of subsequent surgical myotomy or dilatation. However, a number of surgeons have reported, similarly to the situation found after repeated dilations, a more difficult myotomy because of an increased adhesion of muscular layers to mucosal plan with an increased danger of mucosal perforation during the procedure. However, no need of conversion to open approach has been reported in these patients.

PNEUMATIC DILATATION

Historically, the dilation was the first attempt of therapy in esophageal achalasia, described in 1674 by Sir Thomas Willis^[28]. In that case (and in subsequent ones) a whale bone with a sponge tip was successfully used, at least in the short-term. Later, mercury-filled bougies and metal devices were used. More modern dilators consisted of expanding bags or balloons that dilated forcefully the LES, rupturing muscular fibers, like the Syppy pneumatic dilator. The dilators more commonly used today are the polyethylene Microvasive Rigiflex dilator (Boston Scientific Co.), passed over a guided wire, and the Witzel dilator (American Endoscopy) consisting of a polyurethane balloon, mounted over the endoscope.

Forceful dilation of the LES is considered to date to

be the most effective non-surgical treatment for achalasia, although details of the procedure vary in different institutions. The main variables are the type of dilator used, fluoroscopic or endoscopic positioning, and the degree and duration of inflation. We prefer to carry out the procedure under fluoroscopic control in a supine position, as previously described^[29]. Nothing is allowed by mouth for at least 8 h before the procedure. Intravenous midazolam is used for conscious sedation. The dilator is passed over a guidewire, placed endoscopically in the stomach, and positioned across the diaphragmatic hiatus using radiopaque markers as guides. The correct location is assessed by moving the balloon until the waist at inflation of 2-3 psi is observed fluoroscopically in the midportion of the balloon. We start with a 30-mm diameter balloon in the first session; the balloon is inflated to 5 psi during 1 min and subsequently (depending on patient tolerance) to 10-12 psi, and pressure is maintained for another minute. If the procedure is well tolerated, a second session is performed in the consecutive day with a 35-mm balloon, once again in two steps at 5 and 10-12 psi, until the obliteration of the waist occurs. If no symptoms develop during the following 6 h, patients are allowed to eat. If symptoms (fever, chest pain, or cough) or abnormality at chest auscultation occur, a gastrografin swallow is performed. When mucosal tears are observed at the predilation endoscopy, the dilation is postponed for a month.

Overall, a literature analysis of more than 3000 patients showed that the efficacy of pneumatic dilation is 85% in relieving symptoms, with a range of 65%-90%. The average decrease in LES pressure is 54% (range 40%-65%) (V. Annese and G. Bassotti, unpublished data). The results obtained with Rigiflex dilators closely approach those obtained with myotomy, with a perforation rate of about 2%. This is probably the main drawback of pneumatic dilation, which often may require a surgical repair. Risk factors are the use of a large size balloon, and previous dilations. Another drawback of pneumatic dilation is a possible relapse of symptoms, which require additional dilations. This figure is still puzzling, due to large variability between studies and the scanty long-term reports; however, 20%-50% of patients may require additional dilations.

CONCLUSIONS

Achalasia is still an intriguing "mystery", and although different "solutions" are available the definitive "cure" is missing. The variety of therapeutic options may confound patients and doctors; therefore, controlled trials are welcome. These studies, besides the relief of symptoms, should also give information on esophageal emptying, long-term efficacy, patients' satisfaction, cost of procedure, availability of access to referral centers. Unfortunately, it has been estimated that to reach an adequate statistical power a large size trial should be realized, with more than 400 patients enrolled. Waiting for this information, the patients should be informed about all the therapeutic options and institutional experience.

In young patients (under 40 years) or in the presence of a large diameter (> 5-6 cm) oesophagus, a laparoscopic

myotomy is probably the better choice. In the group of patients between 40 and 65 years of age, the graded pneumatic dilation using the Rigiflex balloon should be advised. In elderly patients, poor candidates for surgery, and probably in patients with vigorous achalasia, the initial treatment with botulinum toxin should be the preferred approach. The toxin is also helpful when the pneumatic dilation or myotomy failed^[30] or as a temporary measure.

The usefulness of nifedipine and nitrates is scarce, and these drugs should be used only on a temporary basis, waiting for more effective therapeutic options. In contrast, this pharmacologic approach may be useful on demand in case of severe chest pain.

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Gastric carditis: Is it a histological response to high concentrations of luminal nitric oxide?

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Abstract

During the last decade, inflammation (carditis) and intestinal metaplasia localized to immediately below the human gastro-oesophageal junction have received much attention in relation to the rising incidence of cancer at this site. Since these histological findings are frequently observed even among those who are *H pylori*-negative, the causative factors for such histologic events at the human gastro-oesophageal junction remain obscure. A series of recent studies have demonstrated that a high level of salivary nitrite is sustained over several hours after the ingestion of a high nitrate meal, and that the nitrite in swallowed saliva is rapidly converted to nitric oxide by an acid catalyzed chemical reaction at the gastro-oesophageal junction. Eventually, a substantial amount of nitric oxide diffuses from the lumen into the adjacent tissue. Therefore, the human gastro-oesophageal junction is likely to be a region of high nitrosative stress. Considering the life-time exposure of the gastro-oesophageal junction to cytotoxic levels of nitric oxide, this may account for the high prevalence of inflammation, intestinal metaplasia, and subsequent development of neoplasia at this site. Although gastric acid, pepsin, and bile acid have been intensively investigated as a cause of adenocarcinoma at the gastro-oesophageal junction and the distal esophagus, nitric oxide and the related nitrosative stress should also be examined.

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Key words: Nitric oxide; Gastro-oesophageal junction; Dietary nitrate; Carditis; Intestinal metaplasia at gastro-oesophageal junction

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CARDITIS AND INTESTINAL METAPLASIA AT THE GASTRO-ESOPHAGEAL JUNCTION

The incidence of adenocarcinoma of the cardia of the stomach and adjacent gastro-oesophageal (GO) junction has been rising over the past 25 years, especially in Western countries^[1]. In recent years, a similar trend has been reported in Japan, although GO junction adenocarcinoma is still rare^[2]. We recently reported that this type of cancer occurs in subjects with preserved gastric acid secretion and is not associated with *H pylori* infection^[3]. Although gastric acid, pepsin, and bile have a deleterious effect on the epithelium at that site, the carcinogen for this type of cancer remains unknown. A sequence of histological events in which chronic inflammation progresses to metaplasia, dysplasia and, finally, carcinoma has been documented in distal gastric cancer, and is associated with *H pylori* infection in most cases. The sequence is also well defined for esophageal adenocarcinoma through the formation of intestinal metaplasia (IM) in the distal esophagus (Barrett's esophagus). Although such a process has not been established in the cardia, a similar sequence of events would occur at that site as IM at the squamo-columnar junction which was shown to have a malignant potential similar to that of Barrett's esophagus^[4,5].

During the last decade, inflammation (carditis) and IM localized to the immediate vicinity of the squamo-columnar junction have received much attention in relation to the rising incidence of cancer at this site. These histological findings are reported to be highly prevalent (38%-79% for carditis^[6-10], 10%-36% for IM^[7,11-15]) even among healthy subjects. Of these two histological parameters, the junctional IM is usually associated with carditis^[7,15], and hence the IM could be assumed to be a consequence of chronic inflammation at the cardia as in more distal areas of the stomach^[18]. There are two distinct patterns of carditis^[6,7,9,10]. One is associated with *H pylori* infection and is characterized by inflammation in other sites of the stomach. The other, which is unassociated with *H pylori* infection, comprises inflammation confined to the cardiac mucosa and is seen in patients with otherwise healthy stomachs. There are also differences in the grade and activity of the inflammatory infiltrate involving the columnar mucosa at the squamo-columnar junction, that is, patients with *H pylori*-associated carditis have more severe inflammation than patients with healthy stomachs and neutrophil infiltration is uncommon in the latter group^[6,7,10,17]. Although carditis in *H pylori*-negative subjects is sometimes accompanied by the presence of

gastro-oesophageal reflux disease^[6-10,16,17], its cause remains obscure and it may well represent the histological response to many different types of insult. Considering the milder inflammation usually seen in the absence of neutrophil infiltration, chemical insult can be a probable candidate as a cause of the carditis.

LUMINAL NITRIC OXIDE GENERATION AT THE GASTRO-OESOPHAGEAL JUNCTION

Recently, the hypothesis that a high concentration of luminal nitric oxide (NO) at the human GO junction after nitrate ingestion may be relevant to various diseases at that site was made by a group of researchers in Glasgow^[19,20]. Since then, that group and the present authors have been doing further research on this issue^[21-24].

NO is an important radical that mediates a wide range of physiologic and pathologic events. It is generated at low concentrations by the enzyme constitutive nitric oxide synthase (cNOS) to modulate neuromuscular and vascular functions. Higher concentrations are generated by the inducible form of the enzyme as a part of the immune and inflammatory response. Sustained generation of NO by inducible NO synthase (iNOS) has been implicated in the aetiology of the mutagenesis and neoplasia related to chronic inflammation.

The highest concentrations of NO occurring in the body are not the result of enzymatic synthesis but rather by chemical reactions within the lumen of the stomach, especially in the most proximal area. A previous study reported that high, potentially mutagenic concentrations of NO are generated luminally at the GO junction through the entero-salivary re-circulation of dietary nitrate in humans^[19]. This is explained as follows. Ingested nitrate as an ingredient of food is absorbed from the small intestine, and 25% of this is re-secreted into the mouth by the salivary glands. Bacteria on the dorsum of the tongue then reduce about 30% of this nitrate to nitrite. When salivary nitrite enters the stomach, the combination of the acidity and the ascorbic acid content of the gastric juice converts the nitrite to NO. Since this reaction between nitrite and ascorbic acid at an acidic pH is very rapid^[21], the intraluminal concentration of NO generated by the reaction is maximal at the GO junction and cardia, where the nitrite in saliva first encounters gastric acid. Indeed, this was confirmed by the afore-mentioned study in healthy volunteers which reported that substantial amounts of NO are generated following nitrate ingestion at these anatomical locations, in some case in excess of 50 $\mu\text{mol/L}$ ^[19].

The entero-salivary re-circulation of dietary nitrate is sustained for several hours, during which period the adjacent epithelium of the GO junction is exposed to abundant amounts of NO generated in the lumen. Membranes in the tissues are not barriers to the diffusion of NO because of its gaseous and lipophilic properties. Since NO is known to have dual effects within the tissue, i. e. cytoprotective and cytotoxic depending on the gas level, determination of the NO level is required to evaluate its function in the tissue. We recently developed a rat animal model in which nitrite and acidified ascorbic acid were

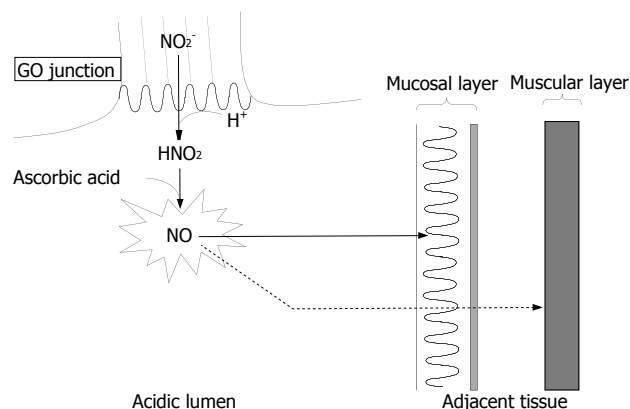


Figure 1 Nitric oxide chemistry at human gastro-oesophageal junction. Nitrite (NO_2^-) in swallowed saliva is converted to nitric oxide (NO) promptly at the gastro-oesophageal (GO) junction where encountering gastric acid containing ascorbic acid. NO thus formed diffuses into the adjacent tissue of the GO junction because of its gaseous and lipophilic properties. The majority of the NO arising from the lumen will be exhausted within the superficial mucosal layer by reacting with surrounding molecules while a small portion of the NO can reach the inner muscular layer. The luminal generation of NO is sustained for several hours after nitrate ingestion, during which period the GO junction is exposed to abundant amounts of NO.

administered separately so that the generation of NO from nitrite would be maximal at the GO junction where the reactants first meet^[24]. In that model, we demonstrated by means of electron paramagnetic resonance spectroscopy with an exogenously supplied NO trapping agent that the NO generated luminally diffused into adjacent tissues to a substantial degree and at a level comparable to that of iNOS derived-NO production and altered the integrity of the surrounding epithelium. As we applied a concentration of nitrite that has been observed in human saliva after the ingestion of a high nitrate meal, this finding may be applicable to the GO junction in humans^[24] (Figure 1).

Briefly, a high level of salivary nitrite is sustained over several hours after the ingestion of a high nitrate meal and the nitrite in swallowed saliva is rapidly converted to NO at the GO junction. Then, a substantial amount of NO eventually diffuses from the lumen into the adjacent tissue. Therefore, the human GO junction is likely to be a region of high nitrosative stress. Considering the life-time exposure of the GO junction to a high level of NO, this may account for the high prevalence of inflammation and IM at that site. Assuming the metaplasia-dysplasia-adenocarcinoma sequence at the GO junction, NO may eventually lead to the development of carcinoma at that site. In addition to being mediated through such a sequence, a high concentration of NO can directly exert a mutagenic and carcinogenic effect through the formation of higher oxides of nitrogen such as N_2O_3 , which can damage DNA directly via deamination of bases and indirectly by forming N-nitrosocompounds. N_2O_3 is also known to inactivate DNA repair enzymes such as O^6 -alkylguanine DNA alkyltransferase^[25-27].

POSSIBLE IMPLICATIONS OF NITRIC OXIDE AT THE GASTRO-OESOPHAGEAL JUNCTION

In this chemical reaction occurring at the GO junction,

the presence of sufficient gastric acid is essential, and this condition is ensured only at the GO junction and the cardia after eating, where the acidic gastric juice largely escapes the buffering effect of food, which decreases the acidity of the gastric juice in the rest of the stomach^[28]. The necessity of gastric acid for luminal NO generation is consistent with our recent clinical study which found that the preservation of gastric acid is necessary for the development of cancer at the GO junction^[3]. In addition, a recent study has demonstrated that the site of NO generation shifted orally to the distal esophagus in cases with gastro-oesophageal reflux^[23]. Hence, the NO thus generated in the distal esophagus may also be relevant to the formation of Barrett's oesophagus and oesophageal adenocarcinoma.

Interestingly, although the majority of NO arising from the lumen would become exhausted within the superficial epithelial layer in our rat model, a small but significant amount of NO was detected even in the inner muscular layer of the GO junction, especially when a high concentration of nitrite was administered^[24]. This suggests that a part of the nitric oxide arising from the lumen could escape from autooxidation occurring within the superficial layer and reach the inner tissue. It is well known that NO endogenously derived from cNOS localized to non-adrenergic, non-cholinergic nerves mediates the relaxation of the smooth muscle cells, including those of the lower esophageal sphincter^[29]. An *in vitro* study using muscle strips from opossum lower esophageal sphincter demonstrated that a tiny amount of NO (in the range of nmol/L) was sufficient to induce relaxation of the muscle^[30]. Therefore, the amount of NO formed in the lumen at the human GO junction may be sufficient to penetrate the epithelium and then affect the inner smooth muscle cell of the lower esophageal sphincter, leading to relaxation of the muscle and reflux of the acidic gastric content into the oesophagus. Actually, a recent report demonstrated in humans that NO generated luminally at the GO junction did affect the lower esophageal sphincter, leading to a significant increase in the transient relaxation of the lower esophageal sphincter^[31]. Further studies are required to clarify to what extent this phenomenon is responsible for the pathogenesis of ordinary gastro-oesophageal reflux disease in humans. However, it may be related at least to the symptoms of heartburn that are known to be invoked after the ingestion of certain foods^[32,33] (Figure 1).

Needless to say, NO is known to have some beneficial effects on the stomach as well. It has been shown that intragastric topical application or parental administration of nitric oxide-releasing substances protects the gastric mucosa from damage induced by injurious agents, suggesting that NO plays an important role in the protection of the gastric mucosa^[34-36]. However, this protective effect is dose-sensitive; as some studies have reported that high doses of exogenous NO can exacerbate gastric mucosal injury^[37,38]. Thus, a high concentration of local NO at the gastric cardia may be involved in the pathology of mucosal injury at that site as a potential cytotoxic mediator. On the other hand, a relatively low

concentration of nitric oxide as seen in the distal stomach may function as a protective mediator to maintain the gastric mucosal integrity. Similarly, ascorbic acid, which is secreted in the healthy human stomach, could show even an undesirable effect on the GO junction by the rapid conversion of salivary nitrite to NO at that site^[21,22], although the vitamin is well known to have beneficial effects as an antioxidant in the remaining stomach.

FUTURE STUDIES ON THE RELEVANCE OF NITRIC OXIDE TO THE VARIOUS DISEASES AT THE GASTRO-OESOPHAGEAL JUNCTION

Further studies to investigate the effect of the diffused NO on the cell biology in the tissue are required to clarify the pathologic relevance of NO to the various histological events occurring at the human GO junction. Accordingly, the characteristic features of NO, for example, the gas is generated abundantly by the unique chemical reaction in the acidic lumen, need to be considered with reference to other examples of pathological circumstances involving NO such as models of inflammation in humans. First, in inflammation, not only NO but other radicals such as superoxides are also generated from inflammatory cells and then the reaction products with NO (for example, peroxynitrite), not NO itself, may be exerting deleterious effects on the tissue^[39]. In contrast, the effect of the diffused NO on the tissue at the GO junction should be mainly an NO-mediated process, at least at the early stage, although once the inflammatory process is initiated by the diffused NO other radicals are also formed. Second, since once the NO is diffused from the acidic lumen into the tissue at neutral pH it will be irreversibly metabolized to nitrite and nitrate, it is the very surface of the epithelium that is exposed to the highest concentration of NO, in contrast with examples of inflammation in which the immediate vicinity of inflammatory cells expressing iNOS would be exposed to the highest levels of endogenous NO^[39]. Hence, the tight junction of the surface epithelium at the GO junction is likely to be the principal target of the NO arising from the lumen^[40,41]. Third, as opposed to the inflammation that occurs only under specific circumstances, the exposure of the human GO junction to localized luminal NO occurs as long as the stomach is healthy and has sufficient acid secretion. In fact, it has been demonstrated that, after the ingestion of nitrate, the luminal NO concentration was maximal at the GO junction in 73% of subjects, although the gas level varied among individuals^[19]. Thus, it appears reasonable that the locally generated NO is the cause of inflammation and IM at the cardia, both of which are frequently observed even in healthy subjects as previously mentioned. However, since neoplasia develops only in a small number of people, individual susceptibility to NO toxicity such as from mutations in cancer-related genes (for example p53) may be important in determining its ultimate contribution to carcinogenesis at the GO junction^[42].

In conclusion, a cytotoxic concentration of NO is generated luminally at the human GO junction and the

gas diffuses into the adjacent tissue. Although gastric acid, pepsin, and bile acid have been intensively investigated as a cause of adenocarcinoma at the GO junction and the distal esophagus, NO and the related nitrosative stress should also be examined.

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GASTRIC CANCER

Risk factors of gastric cancer specific for tumor location and histology in Cali, Colombia

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were not eldest among his/her siblings had an increased risk of GCs in the distal and middle thirds, and their ORs were 1.7 (95% CI 1.0-2.8) and 1.9 (95% CI 0.8-4.3), respectively. The corresponding OR in the upper third stomach was 0.3 (95% CI 0.1-0.9). The differences of those three ORs were statistically significant ($P = 0.010$).

CONCLUSION: The present study shows that birth order, salt intake, consumption of fruits and vegetables, the type of cooking, and cigarette smoking are related to GC risk. In histology and tumor-location specific analyses, non-eldest person among their siblings is related to an increased GC risk in the distal and middle thirds of the stomach, and is related to a decreased GC risk in the cardia.

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Key words: Gastric cancer; Risk factor; Tumor location; Histological type; Colombia

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Abstract

AIM: To examine histology- and tumor-location specific risk factors of gastric cancer (GC).

METHODS: This was a case-control study. The study subjects were 216 GC patients newly diagnosed during the period 2000-2002 and 431 controls selected from non-cancer patients matching in age, gender, and hospital. We obtained information on lifestyles, dietary habits, and others by a questionnaire.

RESULTS: The subjects who were not eldest among his/her siblings were at a slightly elevated GC risk (OR 1.3; 95% CI 0.8-2.0). Salting meals before tasting was related to an increased GC risk (OR 3.5; 95% CI 1.6-7.3). Frequent consumptions of fruits (OR 0.3; 95% CI 0.1-1.0) and vegetables (OR 0.3; 95% CI 0.1-1.0) were related to decreased GC risks. On the other hand, frying foods (OR 1.9; 95% CI 1.0-3.6) and cooking with coal (OR 1.8; 95% CI 1.3-2.6) were related to increased GC risks. Neither Lauren's histological classification (intestinal and diffuse types) nor tumor location significantly affected those associations except birth order. The subjects who

INTRODUCTION

Gastric cancer (GC) is still one of the most common cancers worldwide^[1,2] although its mortality has been decreasing over the years in many countries, including Colombia^[3], due to the changing dietary habits and lifestyles^[1]. Lauren classified GCs into two histological types, i.e., intestinal and diffuse types^[4], and pointed out that intestinal-type GC is more frequent in populations with a high GC incidence while the diffuse-type GC is more frequent in populations with a low incidence^[5]. Lauren suggested the importance of environmental, dietary and socioeconomic factors in development of intestinal-type GCs while he suspected the involvement of genetic factors in diffuse-type GCs.

Correa postulated a multifactorial model for gastric carcinogenesis, in which *H. pylori* infection is considered to play important roles in development of preneoplastic and precursor conditions^[6-8]. Relationships between *H. pylori*

and those gastric lesions have been documented in many countries^[9-12]. Early childhood infection with *H pylori* is considered an important risk factor for GC^[10,13]. A larger sibling size and higher birth order is related to earlier *H pylori* infection in childhood, a condition that is considered an important risk factor for GC^[10,13]. In Colombia, 50% of rural populations are reported to have *H pylori* infection by age 2, and nearly 90% are infected by age 9^[9].

Some studies reported that *H pylori* infection is related to both intestinal- and diffuse-type GCs^[14-16]. Interestingly, microscopic studies suggest that *H pylori* infection seems to be more strongly related to intestinal-type rather than diffuse-type GCs whereas serological studies do not^[16].

Regarding other risk factors, a case-control study in the US, has shown that a high intake of dietary calories increases only intestinal-type GCs^[17]. A population-based prospective study in Japan, reported that the frequent intake of vegetables and fruits, even in low amounts, is associated with reduction of GC risk, and it was observed only in differentiated (intestinal) type^[18]. Another study in Mexico showed that intake of dietary fiber and vitamin E tends to reduce risk of both intestinal- and diffuse-type GCs^[19]. They also reported that the association between high saturated fat consumption and the increase of GC risk is statistically significant among the intestinal type but not in the diffuse type; on the other hand, polyunsaturated fatty acids reduce the GC risk in both intestinal and diffuse types^[19]. The associations of tobacco smoking with different histological types of GCs have been examined by three studies, two conducted in Japan^[20,21] and one in Sweden^[22], to our knowledge. However, none of them showed any evident difference between intestinal and diffuse types with respect to GC risk associated with smoking.

Proximally-located GCs are known to have etiological backgrounds and clinico-pathological features different from more distally located stomach carcinomas^[1]. However, only a small number of studies have examined risk factors of GC by tumor locations. Inoue *et al*^[23] reported that habitual smoking was more strongly associated with the risk of GCs in the upper third of the stomach than that in more-distal parts. However, subsequent studies showed that GCs in the cardia and non-cardia have no evident differences in their associations with smoking^[21,22].

In the present study, we selected factors known, or strongly suspected, to be related to GC risk, and examined whether their associations were affected by Lauren's histological types and tumor location, or not.

MATERIALS AND METHODS

Patients and controls

Cases were GC patients newly diagnosed during the period between Sep 2000 and Aug 2002, in the following three major reference hospitals in Cali, Colombia: Instituto de los Seguros Sociales "Rafael Uribe Uribe", Hospital Universitario del Valle, and Hospital San Juan de Dios. These are reference hospitals not only for cancer but also for non-cancer diseases, including cardiovascular diseases (CVD), metabolic syndromes, trauma, *etc.*

We reviewed medical records, pathological reports and hospital registration records of those hospitals and affiliated medical institutions, including endoscopy clinics and oncology outpatient clinics to obtain information on clinical and pathological diagnosis, disease history, socioeconomic status, and contact address of the GC patients.

We identified 395 GC patients during the study period, and the following 81 cases were excluded: 16 recurrent cases of GCs; 65 patients who lived in Valle del Cauca less than five years. There were 91 patients we could not contact, and 7 patients refused to participate in the study. Among 216 patients in the present study, 30 had died before interview but their relatives accepted to answer the questionnaire and provide the necessary information.

Information on tumor location was obtained from pathological reports and clinical records. The location of a tumor, defined as the predominant location of the tumor, was divided into the following three categories: the upper-third, middle-third and lower-third parts according to the guidelines of the Japanese Research Society for Gastric Cancer^[24]. We could not obtain information on tumor location for 23 cases, and those cases were excluded from the tumor location specific analysis.

We could retrieve formalin-fixed paraffin-embedded blocks of 173 GCs, mainly surgically resected tumors. We examined those specimens, and histological diagnosis was made based on the Japanese classification^[25].

Controls were selected from hospitalized patients diagnosed as neither malignant diseases nor gastric illnesses. Two controls were selected from non-cancer patients for each case, matching in gender, age (5-year category), and hospitals. Controls were selected after their case was interviewed. The mean period of time between the interviews of a case and its controls was 25 wk (range 1-109). Among 528 patients as potential controls, 67 patients were excluded because they had lived in Valle del Cauca less than 5 years. Twenty-nine patients refused to participate in the study, including 19 patients in severe clinical conditions. After all procedures for selection and matching, one patient turned out to be inappropriate as a control because he had been diagnosed as GC 15 years before. Thus, the total number of controls was 431. Four major causes of hospitalization of control patients were cardiovascular diseases ($n = 208$), trauma ($n = 117$), infectious diseases ($n = 38$), and urological disorders ($n = 21$). The Institutional Review Board of the Faculty of Health, Universidad del Valle, Cali, Colombia, approved this study and all subjects gave informed consent.

Interview procedures

The interview was conducted during the period from Sept 2000 to Dec 2002. All subjects were interviewed during hospitalization, except 30 deceased patients. The interviewer visited at houses of those patients' relatives for interview. We used a validated questionnaire to obtain the personal and family information of all subjects as well as lifestyles, dietary intakes, culinary uses, and occupational exposure.

Answers to the questions of fruit and vegetable intakes, the way of cooking and so on were dichotomized

Table 1 Gender specific distribution of age and hospitals *n* (%)

	Male		Female	
	Case	Control	Case	Control
Total	136	271	80	160
Age (yr)				
-49	25 (19)	50 (19)	13 (16)	26 (16)
50-54	18 (13)	36 (13)	7 (9)	14 (9)
55-59	13 (10)	26 (10)	6 (7)	12 (7)
60-64	19 (14)	38 (14)	12 (15)	24 (15)
65-69	16 (12)	32 (12)	14 (18)	28 (18)
70-74	24 (17)	48 (17)	15 (19)	30 (19)
75+	21 (15)	41 (15)	13 (16)	26 (16)
Hospital				
ISS	43 (31)	85 (31)	24 (30)	48 (30)
HUV	66 (49)	132 (49)	38 (48)	76 (48)
HSJD	27 (20)	54 (20)	18 (22)	36 (22)

ISS: Instituto de los Seguros Sociales "Rafael Uribe Uribe"; HUV: Hospital Universitario del Valle; HSJD: Hospital San Juan de Dios.

as follows: "yes" (more than 3 d/wk) and "no" (equal to or less than 3 d/wk). Salt intake was assessed by asking the habit of seasoning a dish with salt before tasting, and the answers were coded as "yes" (usually or some times) and "no" (never). To the question about salting, 4.2% of the cases and 0.9% of the controls answered "sometimes". The simplified dietary habit questionnaire used in the present study was developed on the basis of the validated semi-quantitative food-frequency questionnaire (FFQ) used in a Colombian study in 1998^[26]. The FFQ has been originally developed as Colombian version of the FFQ used in the Nurses Health Study by Willet *et al*^[27]. The validity of our simplified dietary habit questionnaire was confirmed in a study reported by Garcia *et al*^[28].

Statistical analysis

The association between GC risk and each factor was analyzed using conditional logistic regression models. Maximum likelihood estimates of odds ratios (ORs) and corresponding 95% confidence intervals (95% CIs) were calculated. All *P* values presented are two-sided. *P* < 0.05 was taken as significant.

RESULTS

We successfully interviewed 216 GC cases and 431 control patients (Table 1). Table 2 summarizes the gender specific distributions of tumor location and histological classification of GCs. We observed no difference in the distribution of tumor location between male and female cases. Male GCs were more frequently found to be intestinal-type tumors when compared to female cases. Among males, the mean age of cases was highest in the GCs located in the upper-third stomach among the three tumor locations. On the other hand, female cases with tumors in the lower-third stomach showed a relatively higher mean age than those with tumors in other locations. Regarding histological type, female cases with intestinal-type tumors showed a higher mean age than other

Table 2 Clinicopathological features of GC cases (mean ± SD), *n* (%)

	Male	Age	Female	Age
Total	136		80	
Tumor location				
Upper third	16 (12)	65 ± 12	8 (10)	59.0 ± 14.8
Middle third	33 (24)	60 ± 12.8	19 (24)	62.4 ± 13.7
Lower third	74 (54)	61.3 ± 13.9	43 (54)	64.0 ± 13.1
Unknown	13 (10)	60.3 ± 15.5	10 (12)	62.1 ± 13.7
Histological type ¹				
Intestinal	62 (46)	61.4 ± 13.7	24 (30)	66.6 ± 10.1
Diffuse	51 (37)	60.7 ± 14.1	36 (45)	60.3 ± 16.1
Unknown	23 (17)	61.6 ± 13.1	20 (25)	62.5 ± 12.0
Tumor depth				
Mucosa/Submucosa	13 (10)	55.8 ± 15.4	3 (4)	80.3 ± 5.7
Muscular	4 (3)	64.8 ± 8.3	0	-
Serosa	68 (50)	60.6 ± 12.7	31 (39)	61.1 ± 13.5
Unknown	51 (37)	63.5 ± 14.3	46 (57)	63.0 ± 13.0

¹Histological patterns were classified on the basis of Japanese classification as follows: well differentiated tubular adenocarcinoma (tub1), moderately differentiated tubular adenocarcinoma (tub2), solid poorly differentiated adenocarcinoma (por1), non-solid poorly differentiated adenocarcinoma (por2), signet ring cell carcinoma (sig), and mucinous carcinoma (muc). Intestinal type consists of tub1, tub2, and muc, and diffuse type consists of por1, por2, and sig.

subgroups including males.

We examined the association of GC risk with well-known or strongly suspected factors related to GC risk (Table 3). GC risk was related to the habit of seasoning a dish with salt before tasting (*P* = 0.001), the lower frequency of eating fruits (*P* = 0.046) and vegetables (*P* = 0.041), and the higher frequency of frying food consumption (*P* = 0.039), and cooking with coal (*P* < 0.001). The associations were not significantly changed after excluding patients with cardiovascular diseases or trauma from the control series. All those variables that were found to be significantly related to GC risk were independently associated with GC risk in the multivariate analysis involving all those factors (data not shown). The associations of GC risk with occupations and occupational exposures were examined as well. None of them was significantly related to GC risk (data not shown).

We estimated histology specific ORs of GC risk (Table 4). Among those who were not eldest among their siblings, diffuse-type GCs showed a significantly increased risk whereas intestinal-type GCs did not. The observations that non-eldest person among their siblings was related to diffuse-type GC risk but not to intestinal-type GC risk do not necessarily mean that those two histological types have significantly different associations with birth orders. A statistical test addressing this question gave a *P* value larger than 0.05, indicating that observed difference of ORs between intestinal and diffuse types was not statistically significant. When examining potential GC risk factors as shown in Table 3, we found that their ORs did not show any significant differences between the two histological types. On the other hand, smoking was found to be related to diffuse-type GC risk but not to intestinal-type. Here again, however, the difference in the ORs between two

Table 3 Factors related to GC risk- results of conditional logistic analysis

	Case	Control	OR	(95% CI)	P
Birth order ¹					0.123 ²
1	49	125	1	(referent)	
2-3	80	152	1.3	(0.8-2.0)	
4+	85	151	1.4	(0.9-2.2)	
Salting meals before tasting					0.001
No	197	420	1	(referent)	
Yes	19	11	3.5	(1.6-7.3)	
Frequent fruits intake					0.046
No	7	4	1	(referent)	
Yes	209	127	0.3	(0.1-1.0)	
Frequent vegetable intake					0.041
No	8	5	1	(referent)	
Yes	208	426	0.3	(0.1-1.0)	
Steaming foods					0.782
No	172	347	1	(referent)	
Yes	44	84	1.1	(0.7-1.6)	
Frying foods					0.039
No	13	49	1	(referent)	
Yes	203	382	1.9	(1.0-3.6)	
Smoking foods					0.161
No	160	341	1	(referent)	
Yes	56	90	1.3	(0.9-1.9)	
Cooking with oven					0.105
No	158	289	1	(referent)	
Yes	58	142	0.7	(0.5-1.1)	
Cooking with coal					< 0.001
No	100	264	1	(referent)	
Yes	116	167	1.8	(1.3-2.6)	
Roasting foods					0.243
No	74	166	1	(referent)	
Yes	142	265	1.2	(0.9-1.8)	
Cigarette smoking					0.231 ²
				P for heterogeneity = 0.098	
Never	78	188	1	(referent)	
Ex-smoker	89	145	1.5	(1.0-2.3)	
Current smoker	49	98	1.2	(0.8-1.9)	
Cigarettes smoked per day					0.323 ²
(ex-smoker and current smokers combined)				P for heterogeneity = 0.079	
Never	78	188		(referent)	
1-14	93	145	1.6	(1.1-2.3)	
15-24	33	79	1	(0.6-1.7)	
25+	12	19	1.6	(0.7-3.6)	
Cigarette smoking (men only)					0.731 ²
				P for heterogeneity = 0.160	
Never	36	88	1	(referent)	
Ex-smoker	65	104	1.6	(1.0-2.6)	
Current smoker	35	79	1.1	(0.6-1.9)	

¹Information on birth order was missing in 2 cases and 3 controls; ²P for trend.

histological types was not statistically significant.

Table 5 summarizes the results of tumor-location specific analyses. The ORs of GCs in the lower and middle third parts of the stomach were increased among those who were not eldest among their siblings. The ORs were 1.7 and 1.9, respectively. On the other hand, the corresponding OR for GCs in the upper-third stomach was lower than the unity (OR = 0.3). The differences of

ORs among those three tumor locations were statistically significant ($P = 0.010$). When cases with tumors in the lower and middle thirds of the stomach were combined, the OR of non-eldest person was 1.7 (95% CI = 1.1- 2.7, $P = 0.017$). When this OR was compared with that in the upper third of the stomach (OR = 0.3), P value for the difference became smaller and was 0.003. When examining the habit of seasoning a dish with salt before tasting, the low frequency of fruit and vegetable intakes, the frequent consumption of frying foods, and the use of coal for cooking, we found that their ORs were not significantly affected by tumor location.

Smoking was related to the risk of GCs in the upper third of the stomach but not in other locations. A statistical test examining the difference of the ORs in three tumor locations gave a P value larger than 0.05, indicating that the difference was not statistically significant.

DISCUSSION

In the present study, subjects who were not eldest among their siblings were at an elevated risk of GCs in the distal and the middle thirds, indicating a possibility that birth order may be related to age at first *H pylori* infection as suggested by Blaser *et al*^[10]. On the other hand, a decreased OR of GC in the proximal third of the stomach was observed among those who were not eldest among their siblings. Those findings are of interest since *H pylori* infection is suspected to be associated with a decreased risk of GCs in the cardia or proximally located GCs, and with an increased risk of GCs in the non-cardia or distally located GCs^[16]. Studies in the US and Europe showed an inverse relationship between seroprevalence of *H pylori* and GC risk in the cardia^[29,30], suggesting that proximal and distal parts of the stomach may have differences in the interaction between *H pylori* and the host defense system.

We had information about *H pylori* infection for 96 patients. *H pylori* was reported positive in 24% of GCs in the lower third of the stomach ($n = 71$) and 36% of GCs located in the middle part ($n = 17$) whereas none in the tumors located in the upper third of the stomach ($n = 8$). Those findings, albeit small in number, support the notion that *H pylori* is not related to GC risk in the proximally located GCs.

High salt intake produces atrophic gastritis and decreases the acidity of the stomach, creating a condition favoring *H pylori* infection. It is also suspected that *H pylori* infection and high salt intake may act synergistically to promote GC development^[11,12]. If that is the case, it may be expected that salt intake is strongly related to non-cardia cancer but not to the cancer of the cardia (since *H pylori* infection is suspected to increase non-cardia GC risk but decrease cardia GC risk). In the present study, however, the habit of seasoning a dish with salt before tasting was related to an elevated GC risk regardless of histological type or tumor location. It may be because the upper third of the stomach includes not only the cardia but also the non-cardia part of the stomach. We did not have information to identify the cases occurring in the cardia. It is of note that Mayne *et al* examined the effect of sodium intake on tumor-location-specific GC risk and

Table 4 Factors related to GC risk- results of histology specific analysis

	Histological type					
	Intestinal			Diffuse		
	Case	Control	OR (95% CI)	Case	Control	OR (95% CI)
Birth order ¹						
1	21	43	1.0 (referent)	16	58	1.0 (referent)
2+	65	125	1.1 (0.6-2.0) <i>P</i> = 0.876	70	116	2.0 (1.1-3.8) <i>P</i> = 0.026
Salting meals before tasting						
No	79	165	1.0 (referent)	79	172	1.0 (referent)
Yes	7	6	2.3 (0.8-6.9) <i>P</i> = 0.128	8	2	8.0 (1.7-37.7) <i>P</i> = 0.009
Frequent fruit intake						
No	4	1	1.0 (referent)	2	3	1.0 (referent)
Yes	82	170	0.1 (0.01-1.1) <i>P</i> = 0.063	85	171	0.8 (0.1-4.5) <i>P</i> = 0.753
Frequent vegetable intake						
No	4	2	1.0 (referent)	3	2	1.0 (referent)
Yes	82	169	0.2 (0.05-1.4) <i>P</i> = 0.109	84	172	0.3 (0.1-2.0) <i>P</i> = 0.229
Steaming foods						
No	77	138	1.0 (referent)	64	139	1.0 (referent)
Yes	9	33	0.5 (0.2-1.1) <i>P</i> = 0.070	23	35	1.3 (0.8-2.5) <i>P</i> = 0.263
Frying foods						
No	4	14	1.0 (referent)	3	21	1.0 (referent)
Yes	82	157	1.8 (0.6-5.3) <i>P</i> = 0.324	84	153	3.7 (1.1-12.5) <i>P</i> = 0.038
Smoking foods						
No	65	132	1.0 (referent)	63	135	1.0 (referent)
Yes	21	39	1.1 (0.6-2.0) <i>P</i> = 0.792	24	39	1.3 (0.7-2.3) <i>P</i> = 0.374
Cooking with oven						
No	64	110	1.0 (referent)	61	117	1.0 (referent)
Yes	22	61	0.6 (0.3-1.1) <i>P</i> = 0.101	26	57	0.9 (0.5-1.6) <i>P</i> = 0.617
Cooking with coal						
No	37	108	1.0 (referent)	43	101	1.0 (referent)
Yes	49	63	2.2 (1.3-3.8) <i>P</i> = 0.003	44	73	1.4 (0.8-2.4) <i>P</i> = 0.190
Roasting foods						
No	30	70	1.0 (referent)	31	64	1.0 (referent)
Yes	56	101	1.4 (0.8-2.5) <i>P</i> = 0.282	56	110	1.1 (0.6-1.9) <i>P</i> = 0.841
Cigarette smoking						
Never	32	72	1.0 (referent)	30	88	1.0 (referent)
Ex-smoker	37	58	1.5 (0.8-2.6)	35	52	2.1 (1.1-4.0)
Current smoker	17	41	0.9 (0.4-1.9)	22	34	2.0 (1.0-4.2)
<i>P</i> for trend = 0.931				<i>P</i> for trend = 0.027		
<i>P</i> for heterogeneity = 0.340				<i>P</i> for heterogeneity = 0.032		

¹Information on birth order was missing in a patient with diffuse-type GC and in 3 controls.

showed that the OR of GCs was 1.31 (95% CI = 0.86-2.00) in the cardia and was 1.46 (95% CI = 1.00-2.15) in the non-cardia^[31].

The tumor-location specific analyses showed that smoking appeared to be more strongly related to tumors in the upper third of the stomach. However, the difference

in the magnitude of associations was not statistically significant as was the case with histological type. A recent Japanese study showed that the association of smoking with GC in the upper third of the stomach was only slightly weaker than that with GC in the middle and lower thirds of the stomach^[21]. A Swedish study by Ye *et*

Table 5 Factors related to GC risk -- results of tumor-location specific analysis

	Tumor location								
	Lower third			Middle third			Upper third		
	Case	Control	OR (95% CI)	Case	Control	OR (95% CI)	Case	Control	OR (95% CI)
Birth order ¹									
1	26	77	1.0 (referent)	8	27	1.0 (referent)	10	8	1.0 (referent)
2+	90	155	1.7 (1.0-2.8)	44	75	1.9 (0.8-4.3)	14	40	0.3 (0.1-0.9)
			<i>P</i> = 0.054			<i>P</i> = 0.154			<i>P</i> = 0.028
Salting meals before tasting									
No	106	228	1.0 (referent)	47	101	1.0 (referent)	22	47	1.0 (referent)
Yes	11	6	3.7 (1.4-9.9)	5	2	5.0 (1.0-25.8)	2	1	4.0 (0.4-44.1)
			<i>P</i> = 0.010			<i>P</i> = 0.054			<i>P</i> = 0.258
Frequent fruits intake									
No	5	3	1.0 (referent)	2	1	1.0 (referent)	0	0	-
Yes	112	231	0.3 (0.1-1.3)	50	102	0.3 (0.02-2.7)	24	48	-
			<i>P</i> = 0.099			<i>P</i> = 0.258			-
Frequent vegetable intake									
No	6	2	1.0 (referent)	1	1	1.0 (referent)	0	1	-
Yes	111	232	0.2 (0.03-0.8)	51	102	0.5 (0.03-8.0)	24	47	-
			<i>P</i> = 0.028			<i>P</i> = 0.624			-
Steaming foods									
No	92	190	1.0 (referent)	42	83	1.0 (referent)	21	40	1.0 (referent)
Yes	25	44	1.2 (0.7-2.1)	10	20	1.0 (0.4-2.3)	3	8	0.7 (0.2-3.0)
			<i>P</i> = 0.564			<i>P</i> = 1.00			<i>P</i> = 0.639
Frying foods									
No	6	30	1.0 (referent)	3	9	1.0 (referent)	1	2	1.0 (referent)
Yes	111	204	2.6 (1.1-6.2)	49	94	1.6 (0.4-6.0)	23	46	1.0 (0.09-11.02)
			<i>P</i> = 0.038			<i>P</i> = 0.525			<i>P</i> = 1.000
Smoking foods									
No	90	183	1.0 (referent)	35	80	1.0 (referent)	17	38	1.0 (referent)
Yes	27	51	1.1 (0.7-1.8)	17	23	1.7 (0.8-3.6)	7	10	1.5 (0.5-4.2)
			<i>P</i> = 0.791			<i>P</i> = 0.173			<i>P</i> = 0.468
Cooking with oven									
No	83	154	1.0 (referent)	37	73	1.0 (referent)	19	30	1.0 (referent)
Yes	34	80	0.8 (0.5-1.3)	15	30	1.0 (0.5-2.1)	5	18	0.4 (0.1-1.4)
			<i>P</i> = 0.301			<i>P</i> = 1.000			<i>P</i> = 0.153
Cooking with coal									
No	57	141	1.0 (referent)	20	63	1.0 (referent)	11	31	1.0 (referent)
Yes	60	93	1.6 (1.0-2.6)	32	40	2.2 (1.2-4.2)	13	17	2.3 (0.8-6.9)
			<i>P</i> = 0.037			<i>P</i> = 0.014			<i>P</i> = 0.109
Roasting foods									
No	46	89	1.0 (referent)	16	43	1.0 (referent)	6	15	1.0 (referent)
Yes	71	145	0.9 (0.6-1.5)	36	60	1.8 (0.8-3.8)	18	33	1.3 (0.5-3.9)
			<i>P</i> = 0.798			<i>P</i> = 0.131			<i>P</i> = 0.597
Cigarette smoking									
Never	41	107	1.0 (referent)	20	44	1.0 (referent)	5	23	1.0 (referent)
Ex-smoker	47	68	1.9 (1.1-3.4)	22	41	1.2 (0.6-2.5)	13	15	3.7 (1.1-12.5)
Current smoker	29	59	1.3 (0.7-2.3)	10	18	1.3 (0.5-3.4)	6	10	3.0 (0.6-13.9)
			<i>P</i> for trend = 0.257			<i>P</i> for trend = 0.597			<i>P</i> for trend = 0.083
			<i>P</i> for heterogeneity = 0.059			<i>P</i> for heterogeneity = 0.859			<i>P</i> for heterogeneity = 0.070

¹Information on birth order was missing in a patient with tumor in the lower third of the stomach and in 3 controls.

et al.^[22] also reported that smoking was related not only to cancer of the cardia but also to cancer of the non-cardia. Both studies mentioned above also showed no evident difference of smoking-related GC risk between the two histological types.

Frequent intakes of fruits and vegetables were related to a decreased GC risk in the present study. No significant difference in magnitude of their associations with respect to histological type and tumor location was

observed, confirming what was reported by other previous studies^[17,32,33]. Although the mechanisms involved in the protective effects of frequent fruit and vegetable intakes on GC risk are yet to be elucidated, Yuasa *et al.*^[34] showed the intake of some specific vegetables may prevent hypermethylation of the CDX2 gene, which upregulates the expression of MUC2, an important mucin protein related to the intestinal phenotype of GCs.

We observed a significant association between GC risk

and frying foods. In Cali, Colombia, residents tend to fry starch-rich foods, including potatoes, green banana, corn, and mandioca. De Stefani *et al*^[35] reported a relationship between starchy rich foods and the GC risk, suggesting mechanical damages to the gastric mucosa and promotion of nitrosation by low-protein diets. Mayne *et al*^[31] suggested that this association may be related with micronutrient deficiencies.

We also observed an association between cooking with coal and the GC risk. It is known that barbecued foods contain high concentrations of N-benzil pyrene and nitrites, which are related to an increased GC risk^[11,21]. Although some studies suggest that cooking meats at a very high temperature produces nitrosamines and mutagen substances^[36,37], further studies are required to confirm this association.

Taken together, well-known and strongly-suspected risk factors of GCs did not show any statistically significant predisposition to particular histological types or tumor location. An exception was the finding that the non-eldest person was related to an increased risk of GCs in the distal and middle third of the stomach, and was associated with a decreased cancer risk in the proximal third of the stomach.

One of the explanations for the absence of difference in other risk factors was a lack of statistical power. It may be particularly so with respect to tumor location; the number of cancer cases in the upper third of the stomach in the present study may be too small to find any differences. That may also be true for the lack of difference in the risk factors for two histological-type GCs. However, another explanation may also be possible; those findings in the present study, together with the observations that *H pylori* infection is related to both intestinal- and diffuse-type GCs, may be casting a doubt on the hypothesis that intestinal- and diffuse-type GCs are distinctly different in their etiological backgrounds.

Intestinal and diffuse types of Lauren's classification correspond, in principle, to well-differentiated and poorly differentiated ones of Sugano and Nakamura^[38,39], respectively. It is of note, here however, that Lauren's classification is created by studying advanced tumors while the classification of Nakamura and Sugano is obtained from analysis of early carcinomas. Nakamura^[39] reported that 85% of micro carcinomas of the stomach (< 5 mm) were of well-differentiated type, and, conversely, the proportion of undifferentiated type was higher in larger tumors; thus such a proportion increased according to tumor size. On the basis of mucin phenotype expression of GCs, Tatematsu *et al*^[40] have postulated that GC starts as gastric-phenotype carcinomas, expressing mucin that is only expressed in gastric membranes, and during development into large carcinomas, some tumors express mucins whose expressions are specific for intestinal membranes. They pointed out a possibility of intestinal metaplasia accompanying GCs, particularly intestinal-type GCs, unrelated to GC development.

In conclusion, the present study showed that birth order, salt intake, consumptions of fruits and vegetables, the type of cooking, and cigarette smoking are related to GC risk. In histology and tumor-location specific

analyses, non-eldest person among their siblings is related to an increased GC risk in the distal and middle thirds of the stomach, and is related to a decreased GC risk in the cardia. Those findings may help understand the mechanisms of the GC development in different locations of the stomach. Further studies seem warranted.

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GASTRIC CANCER

Inhibitory effect of arsenic trioxide on angiogenesis and expression of vascular endothelial growth factor in gastric cancer

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Abstract

AIM: To investigate the inhibitory effect of As₂O₃ on angiogenesis of tumor and expression of vascular endothelial growth factor (VEGF) in tumor cells *in vivo* and *in vitro*.

METHODS: The solid tumor model was formed in nude mice with the gastric cancer cell line SGC-7901. The animals were randomly divided into three groups. As₂O₃ was injected into the arsenic-treated groups (2.5 mg/kg and 5 mg/kg) and the same volume of saline solution was injected into the control group. Microvessel density (MVD) and expression of VEGF were detected with immunofluorescence laser confocal technology. Further expression of VEGF protein and VEGF mRNA was measured with Western blotting and fluorescence quantitative RT-PCR in SGC-7901 cells treated with As₂O₃.

RESULTS: In nude mice, after treatment with 5 mg/kg and 2.5 mg/kg As₂O₃ respectively, about 50% and 30% tumor growth inhibition were observed correspondingly ($P < 0.05$, $P < 0.05$). Decrease in MVD appeared in As₂O₃-treated tumors compared with control group ($P < 0.001$, $P < 0.001$). MVD in tumors was significantly lower in 5 mg/kg group than in 2.5 mg/kg group ($P < 0.01$). The fluorescence intensity levels of VEGF in tumor cells were significantly lowered in the arsenic-treated groups ($P < 0.01$, $P < 0.01$). The fluorescence intensity level of VEGF in 5 mg/kg group was lower than that in 2.5 mg/kg group ($P < 0.01$). *In vitro*, the expression of VEGF

protein decreased in dose- and time-dependent manner after the treatment with As₂O₃, but in VEGF mRNA no significant difference was found between the control group and the treated groups.

CONCLUSION: As₂O₃ can inhibit solid tumor growth by inhibiting the formation of new blood vessels. One of the mechanisms is that As₂O₃ can inhibit VEGF protein expression.

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Key words: Arsenic trioxide; Vascular endothelial growth factor; Angiogenesis; Tumor growth inhibition

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INTRODUCTION

Arsenic has been used since ancient times as a therapeutic agent. However, until recently its use in modern medicine has been restricted to the treatment of a limited number of parasitic infections. Since the early 1990s, arsenic trioxide (As₂O₃) has been used successfully in the treatment of patients with relapsed and refractory acute promyelocytic leukemia (APL)^[1,2]. As₂O₃ has been shown to cause degradation of PML-RAR alpha, promoting differentiation^[3,4]. However, degradation of PML-RAR alpha may not be wholly responsible for the great sensitivity of APL cells to As₂O₃. Other investigations subsequently found that As₂O₃ can induce apoptosis of APL cells, other malignant myeloid, lymphoid, and megakaryocytic cells.

The mechanism by which arsenic exerts effect of inhibiting tumor still remains uncertain. Investigations of arsenic have found that its efficacy is dependent upon a number of mechanisms. Arsenic may affect numerous intracellular signal transduction pathways and causes many alterations in cellular function^[5-8]. Recently, it has been reported that As₂O₃ can decrease microvessel density and inhibit angiogenesis in solid tumors^[9-11], but its pathway is

not clear.

Angiogenesis is an important factor in the progression and enlargement of solid neoplasms and is in close relation to invasion and metastases^[12-14]. Angiogenesis can be divided into a series of temporally regulated responses, including protease induction, endothelial cell migration, proliferation and differentiation^[15]. This process begins with the production and release of angiogenic factors by endothelial cells, tumor cells and matrix cells. Vascular endothelial growth factor (VEGF) produced by tumor cells is a potent angiogenic factor, and it binds to endothelial cell surface receptors and activates various functions of the cells^[16-19]. Therefore, angiogenesis can be reflected by the expression of VEGF.

In the present report, we investigated the inhibitory effect of As₂O₃ on angiogenesis of tumor and expression of VEGF in tumor cells *in vivo* and *in vitro*. The solid tumor model was formed in nude mice with the gastric cancer cell line SGC-7901. Microvessel density (MVD) and expression of VEGF were detected by immunofluorescence laser confocal technology. *In vitro* we measured further expression of VEGF protein and VEGF mRNA with Western blots and real-time fluorescence quantitative RT-PCR.

MATERIALS AND METHODS

Animals and cells

Thirty 5-wk-old male BALB/C-nu/nu mice weighing 19-21 g were purchased from Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, China. Mice were acclimatized at the Animal Laboratory Centre of the Fourth Military University for 1 wk before receiving injections of cancer cells. The mice were kept in a laminar-filtered airflow cabinet under pathogen-free conditions with a constant temperature of $22 \pm 2^\circ\text{C}$, relative humidity of $55\% \pm 5\%$ and 12-h dark/light cycles. All experiments were carried out according to the guidelines of the Laboratory Protocol of Animal Handling of the Fourth Military Medical University. Human gastric cancer cell line, SGC-7901, was purchased from the Animal Laboratory Centre of the Fourth Military Medical University. SGC-7901 cells were cultured in RPMI 1640 medium supplemented with 100 mL/L fetal bovine serum (FBS) at 37°C in a 50 mL/L CO₂ incubator.

Drug and instruments

Arsenious acid [$\text{H}_3\text{As}_2\text{O}_3$ ($\text{As}_2\text{O}_3 + 3\text{H}_2\text{O} \rightleftharpoons \text{H}_3\text{As}_2\text{O}_3$)] was supplied by Yida Pharmaceutical Co, Harbin, China. VEGF expression in tumor xenografts was examined by a TCS SP2 laser confocal microscope made by LEICA Cc, Germany. VEGF mRNA was examined by real time system ABI7000 (Applied Biosystems, Foster City, CA, USA).

Xenografts in nude mice and treatment

Thirty mice received sc injection into the right flanks with a 200 μL cell suspension containing 2×10^7 SGC-7901 cells. After 10 d, when established tumors of 0.2-0.3 cm³ diameter were detected, drug administration was started. The animals were randomly divided into three groups consisting of 10 animals in each. Arsenious acid diluted with 9 g/L saline solution was injected into the peritoneal cavity

every day to the two treatment groups (2.5 mg As₂O₃/kg and 5 mg As₂O₃/kg in 0.2 mL) and the same volume of 9 g/L saline solution was injected to the control group. After 10 d of treatment, the three groups of mice were sacrificed and the tumor masses were removed. After the weights of tumor mass were measured, the tumor mass was fixed in 40 g/L paraformaldehyde and frozen sections prepared with a cryostat (HM505E Cryomicrom, Germany) for immunofluorescence analysis. The liver and kidneys were prepared for paraffin sections routinely for pathological examination.

Inhibition of tumor growth

Tumor growth inhibition (TGI) was used to assess the therapeutic efficacy against xenografts. The tumor weight was to calculate TGI, that is, $\text{TGI} (\%) = (1 - W_t/W_c) \times 100\%$, where W_t is the mean tumor weight of the arsenic-treated group, W_c is the mean tumor weight of the control group. Tumor growth inhibition was expressed by tumor volumes that were measured before treatment and 6 and 11 d after treatment respectively. Tumor volume expressed as cm³ is calculated using the formula: tumor volume = $1/2ab$, where a is the long axis, b is the short axis. $\text{TGI} (\%) = (1 - V_t/V_c) \times 100\%$, where V_t is the mean tumor volume of the arsenic-treated group, V_c is the mean tumor volume of the control group.

Immunofluorescence examination

CD31 antigen was used as an immunofluorescence marker of vessel endothelial cells. For the immunofluorescent staining of CD31 and VEGF, the frozen sections were kept at room temperature for 30 min, incubated in distilled water for 5 min and in PBS for 5 min, permeabilized in 1 g/L Triton-X-100 for 10 min, washed with PBS (5 min \times 3), blocked with 100 mL/L sheep serum (Sgima) at 37°C for 20 min, incubated in the primary antibody, rat anti-mouse CD31 (Biolegend, USA) or rabbit anti-human VEGF polyclonal antibody (Labvision, USA) at 4°C overnight, washed with PBS (5 min \times 3), incubated in the secondary antibody (goat anti-rat IgG conjugated TRITC or sheep anti-rabbit IgG conjugated FITC, Sigma) for 1 h at 37°C , washed with PBS (10 min \times 3) and then examined under a TCS SP2 laser confocal microscope.

For each group, several field images of VEGF in each tumor tissue section were captured under confocal microscope, fluorescence intensity of each section in the confocal fluorescence images was measured using the Leica Confocal analysis system. The mean fluorescence intensity in each section was then calculated.

Assessment of microvessel density

Microvessel density (MVD) was determined under a confocal microscope in the area of tumor tissue sections. The microvessels were carefully counted on 20 fields ($\times 400$). The mean and SE were expressed as the number of microvessels identified within the area.

Cell culture and administration of As₂O₃ in vitro

SGC-7901 cells were routinely grown in plastic tissue culture flasks in RPMI 1640 medium with 100 mL/L FBS, 100 KU/L penicillin and 100 mg/L streptomycin and

were cultured at 37°C in a 50 mL/L CO₂ humidified atmosphere. SGC-7901 cells in logarithmic growth phase were inoculated in culture medium, without adding (control group) and adding As₂O₃ according to different concentrations of below 0.5, 1, 2 and 3 μmol/L. The tumor cells were collected respectively at 24 h, 48 h and 72 h for Western blot of VEGF. The fluorescent quantitative RT-PCR of VEGF was analyzed in the tumor cells collected at 48 h.

Western blotting for VEGF

After treatment with As₂O₃, SGC-7901 cells were washed with ice-cold PBS and dissolved with lysis buffer (1 mmol/L Na₃VO₄, 0.017 g/L aprotinin, 2 mmol/L PMSF, 25 μmol/L P-N-P, 0.1 g/L leupeptin, 10 g/L NP-40, 0.15 mol/L NaCl, 0.02 mol/L Tris HCl, 0.1 g/L NaN₃). The protein lysates were centrifuged at 12000 r/min for 10 min at 4°C. The protein lysates were mixed with 5 × sample buffer, boiled for 10 min, and analyzed by 120 g/L sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the gel was transferred to a 0.45-μm pure nitrocellulose membrane. The membrane was blocked with 5 mL/L skim milk in TBS-Tween 20 for 1 h at room temperature, and reacted with mouse anti-human VEGF monoclonal antibody (Santa Cruz) at 4°C overnight. The membrane was then incubated with the secondary antibody (rabbit anti-mouse IgG). The protein content was visualized using HRP-conjugated secondary antibodies, followed by enhanced chemiluminescence (ECL).

Fluorescence quantitative RT-PCR for VEGF

RT-PCR for VEGF, total RNA was extracted from tumor cells using Trizol reagent according to the manufacturer's protocol (Promega, Madison, WI, USA). RNA was reversely transcribed to cDNA by the kit (Promega, Madison, WI, USA). Prime and probe were designed with the assistance of Primer Express 2.0. MGB-TaqMan probe of VEGF (VEGF-FAM): 5'-ACCATGCAGATTATG-3'. For VEGF, the following primers were used: 5'-GCCCACTGAGGAGTCCAACA-3', 5'TGGCCTTG-GTGAGGTTTGAT-3'. MGB-TaqMan probe of β-actin (HBA-TAMRA-FAM): 5'CCAGGGCGTGATGGTGG-GCAT 3'. For β-actin the following primers were used: HBA-TAMRA-FP 5'CCGTCTTCCCCTCCATCG 3' HBA-TAMRA-RP 5'GTCCCAAGTTGGTGACGATGC 3'. The primers and probes were synthesized by Shanghai GeneCore BioTechnologies Co., Ltd. All PCR reactions were performed on a real time system ABI7000 (Applied Biosystems, Foster City, CA, USA). The thermal cycle conditions comprised 2 min at 50°C, 10 min at 95°C, 30 s at 95°C and 30 s at 60°C. Each PCR run included five points of calibration curve (5 samples were obtained by tenfold serially dilutions of standard product, and the standard curve was established from the 5 samples for the determination of the relative quantity of genes), a non-template control, and the respective sample cDNA.

Statistical analysis

The data were represented as mean ± SD. The data was analyzed with statistical software of SPSS10.0. Multiple statistical comparisons were performed using ANOVA in

Table 1 Tumor growth inhibition (TGI) of tumors treated with arsenic (*n* = 10, mean ± SD)

Group	Tumor volume (V) mm ³			Mass (mg)	TGI (%)	
	D 0	D6	D11		D6	D11
Control	67 ± 45	343 ± 160	999 ± 338	784 ± 185		
2.5 mg/kg	63 ± 36	307 ± 80	696 ± 125 ^a	556 ± 211 ^a	11.7%	30.3% (29.1%)
5 mg/kg	66 ± 48	240 ± 203	491 ± 116 ^{a,c}	375 ± 167 ^{a,c}	30.0%	50.9% (52.2%)

^a*P* < 0.05 vs control; ^c*P* < 0.05 vs 2.5 mg/kg; TGI calculated using tumor volume was given in parenthesis.

a multivariate linear model. Student's *t* test was used to assess differences between the treatment group and control group. *P* < 0.05 was considered statistically significant.

RESULTS

Inhibition of tumor growth by arsenic trioxide

All nude mice developed tumor after 10 d in implantation of SGC-7901 cells. When drug administration was started, there was no significant difference in the tumor volume of the three groups (*P* > 0.05). The increase in tumor volume and weight in the three groups from d 0 to 11 after treatment is shown in Table 1. Tumor volume growth of 2.5 mg/kg or 5 mg/kg of the arsenic-treated group was reduced by 30.3% or 50.9% after 10 d administration of arsenic trioxide. Tumor weight growth was inhibited by 29.1% and 52.2% respectively in the 2.5 mg/kg and 5 mg/kg arsenic -treated group. There were statistical differences in the tumor volumes and weights in the arsenic -treated group (2.5 mg/kg group and 5 mg/kg group) and control (*P* < 0.05). On the other hand, the tumor volumes and weights in the 5 mg/kg group were significantly less than that in 2.5 mg/kg group (*P* < 0.05). No obvious toxic effects attributable to As₂O₃ therapy were noted. The body weight of the mice was not affected. All of the 30 studied mice were alive at the end of treatment. HE staining of the nude mice's liver and kidney did not reveal pathological changes.

Inhibition of tumor angiogenesis by arsenic trioxide

Sections of tumors from the mice in all three treatment groups were stained for CD31 immunofluorescence to detect the number of endothelial cells (ECs) as a measure of tumor angiogenesis. A single microvessel was defined as any immunofluorescence stained endothelial cell distinguished from adjacent tumor cells and other connective tissue elements. Microvessel density as a parameter of angiogenic activity was decreased in tumors of experimental groups treated with 2.5 mg/kg (Figure 1B) and 5 mg/kg arsenic trioxide (Figure 1C). Microvessel density of arsenic-treated groups was significantly lower than that of the control group (Figure 1A). Significantly lower microvessel density was present in the 5 mg/kg group than that in the 2.5 mg/kg group. These studies demonstrated the decreased capillary density of the tumor with treatment of As₂O₃ (Table 2).

Effect of arsenic trioxide on expression of VEGF in vivo

Expression of VEGF was confirmed by the presence

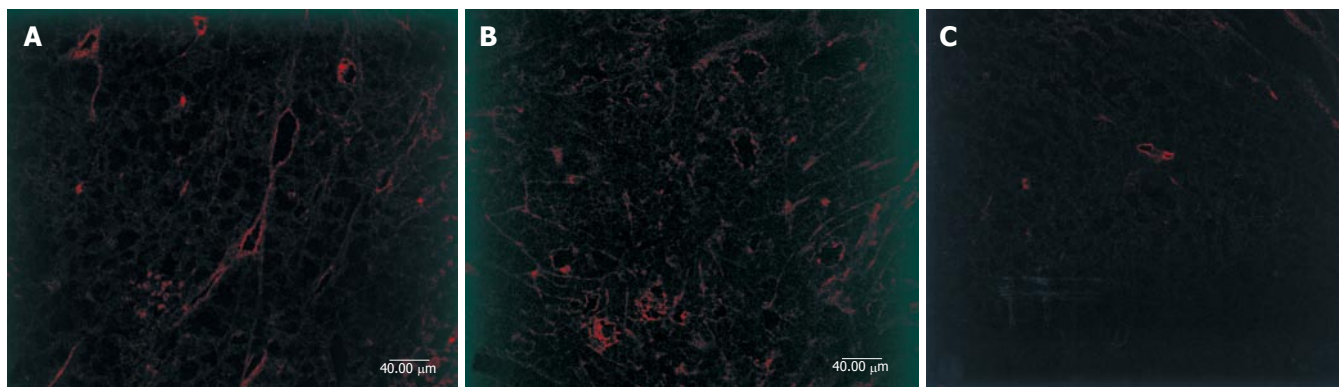


Figure 1 Immunofluorescence staining for microvessel (CD31) in xenograft tumor. Microvessel or endothelial cells were identified by fluorescence staining. Original magnification $\times 400$. **A**: Control group; **B**: 2.5 mg/kg group; **C**: 5 mg/kg group.

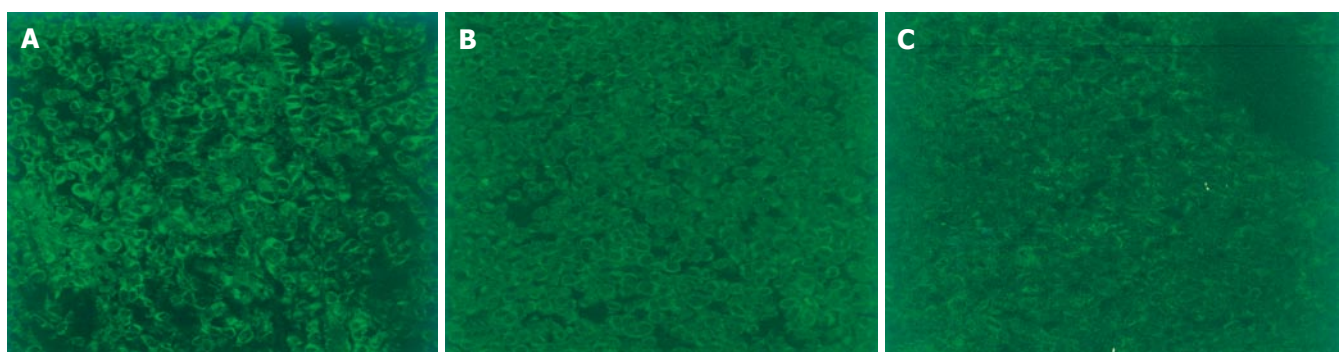


Figure 2 VEGF expression in xenograft tumor. Original magnification $\times 400$. **A**: Control group; **B**: 2.5 mg/kg group; **C**: 5 mg/kg group. The fluorescence expression of VEGF in tumor cells were decreased in the arsenic-treated groups.

Table 2 Effect of As_2O_3 on microvessel density and VEGF expression in tumor xenografts ($n = 10$, mean \pm SD)

Group	MVD	VEGF
Control	9.32 ± 0.33	49.23 ± 2.12
2.5 mg/kg	5.36 ± 0.32^b	20.07 ± 1.19^b
5 mg/kg	$3.05 \pm 0.24^{b,d}$	$11.93 \pm 1.07^{b,d}$
F	111.841	163.461
P	< 0.001	< 0.001

^b $P < 0.01$ vs Control; ^d $P < 0.01$: vs 2.5 mg/kg.

Table 3 Effect of As_2O_3 on VEGF mRNA in SGC-7901 cell by real time RT-PCR

As_2O_3 (μ mol/L)	VEGF mRNA	β -actin mRNA	VEGF mRNA/ β -actin mRNA
0	1.28×10^6	3.72×10^7	3.44×10^{-2}
0.5	2.34×10^4	7.92×10^5	2.95×10^{-2}
1.0	4.74×10^2	9.96×10^3	4.76×10^{-2}
2.0	2.16×10^6	7.24×10^7	2.89×10^{-2}
3.0	3.21×10^3	8.2×10^4	3.91×10^{-2}

of fluorescence-stained cytoplasm in the tumor cells. It is shown in Figure 2, strong immunoreactivity to VEGF was found in all the SGC-7901 tumor xenografts of the control group (Figure 2A). The weaker fluorescence intensity expression was observed in SGC-7901 tumor of the groups treated with 2.5 and 5 mg/kg arsenic trioxide (Figure 2B and C). The fluorescence intensity levels of VEGF in tumor cells were significantly lower in the arsenic-treated groups than in control group ($P < 0.01$). The fluorescence intensity level of VEGF in 5mg/kg group was lower than that in 2.5 mg/kg ($P < 0.01$, Table 2).

Effect of arsenic trioxide on expression of VEGF mRNA and protein in SGC-7901 cell line *in vitro*

To further analyze the effect of arsenic trioxide on VEGF expression in tumor, we assessed VEGF expressions of

SGC-7901 cells *in vitro* using Western blotting and fluorescent quantitative RT-PCR. Western blot analysis revealed that the expression of VEGF protein decreased in a dose- and time-dependent manner after treatment with As_2O_3 (Figure 3). But VEGF mRNA expression was not in agreement with the VEGF protein expression results, VEGF mRNA data did not reveal a significant difference between the control group and the treatment groups (Table 3, Figures 4 and 5).

DISCUSSION

The development of a growing tumor requires an abundant blood supply. Angiogenesis is an important factor in the progression and enlargement of solid neoplasms and close relation to invasion and metastases^[12-14]. Vascularisation

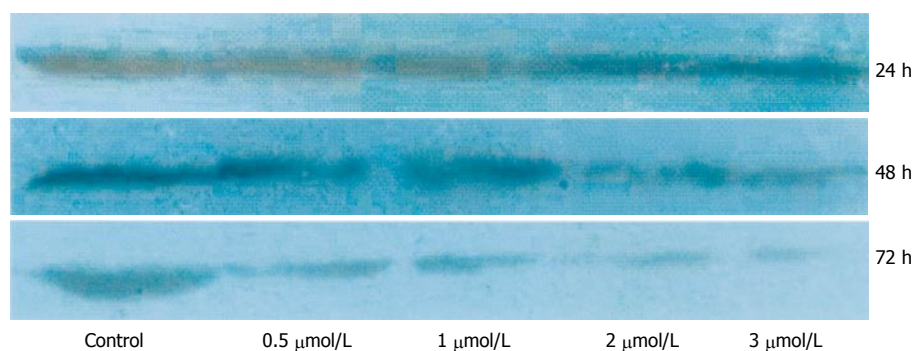


Figure 3 Effect of As_2O_3 on expression of VEGF in SGC-7901 cells by Western blots Analysis.

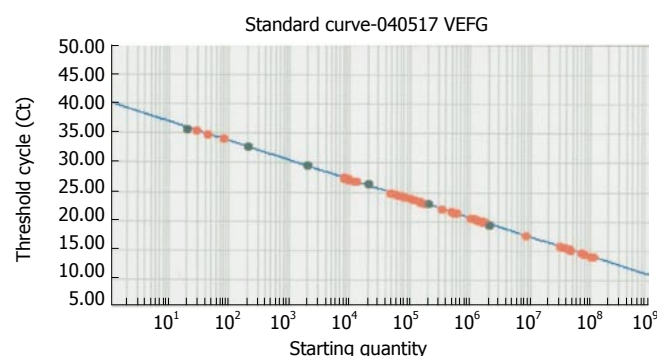


Figure 4 Standard Curve of VEGF.

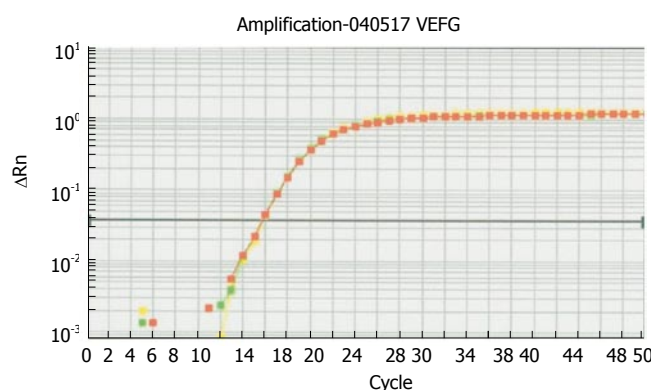


Figure 5 Amplification Curve of VEGF mRNA in SGC-7901.

tion within the primary tumor has a direct relation with the tumor growth rate. Inhibition of angiogenesis may lead to control of tumor growth and metastasis, therefore, antiangiogenesis is a promising therapeutic approach for treatment of cancer^[20-22]. The results presented here indicated that the As_2O_3 is active against angiogenesis *in vivo* and *in vitro*. We examined the effect of As_2O_3 on angiogenesis in a human tumor model in nude mice. The result showed that subcutaneous injection of SGC-7901 cells yielded a 100% tumor uptake rate. About 50% and 30% SGC-7901 tumor growth inhibition was following treatment of As_2O_3 with 5 mg/kg and 2.5 mg/kg separately.

We measured the MVD in tumor tissues by labeled CD31, which marked endothelial cells of microvessels. Decrease of the MVD in tumor tissue was related to treatment of As_2O_3 . A high degree of tumor angiogenesis was observed in mice without treatment of As_2O_3 , but MVD was low in As_2O_3 -treated tumors. There was a significant difference in MVD between the control group and treatment groups. MVD was significantly lower in the high dosage group with 5 mg/kg As_2O_3 than in the low dosage group with 2.5 mg/kg. Our observation was in agreement with the results of Shen^[9]. This study demonstrated that As_2O_3 induced a dose-dependent decrease of MVD, accompanied by inhibition of tumor growth. These suggest that induction of angiogenesis is an important step in carcinogenesis and As_2O_3 inhibits tumor growth by, at least in part, decreasing intratumor MVD.

It has been reported that neovascularization is regulated by accelerators, such as vascular endothelial growth

factor (VEGF), basic fibroblast growth factor (FGF), hepatocyte growth factor (HGF), thymidine phosphorylase, endothelin, IL-4, and IL-8, and by inhibitors such as thrombospondin, endostatin, angiostatin, transforming growth factor β 1 (TGF- β 1), tumor necrotic factor (TNF) and IL-6^[23]. Of these factors, VEGF is involved very much in the proliferation and metastasis of various cancers. There is evidence that the degree of several surrogate angiogenic markers such as microvessel density or (VEGF) expression levels in primary tumor tissue can reflect the biological aggressiveness of tumors and provide prognostic information^[18,23-25].

The VEGF gene family currently includes six members: VEGF-A (prototype VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor. Of them, vascular VEGF, also known as vascular permeability factor, is a potent angiogenic cytokine that induces mitosis and also regulates permeability of endothelial cells. In addition, VEGF prolongs the survival of new blood vessels by inducing the expression of Bcl-2 protein. VEGF plays a crucial role in vasculogenesis and angiogenesis, and is closely related to invasion and metastases of tumors^[26,27].

Several studies reported that the degree of VEGF expression in tumors correlated positively with the level of MVD, the degree of malignancy, stage of tumor, presence/absence of peritoneal dissemination and metastases, and correlated negatively with prognosis^[12,23,28]. In the present study, high expression VEGF existed in tumor tissues without treatment of As_2O_3 which also showed an increase of MVD, suggesting that VEGF is

most closely associated with induction and maintenance of the neovasculature in human gastric cancer. Since human gastric cancer SGC-7901 cells secrete high amounts of VEGF and develop in nude mice tumors whose growth is highly VEGF-dependent, they provide a good model to test the availability of molecules that inhibit VEGF bioactivity.

In recent years, it has been widely shown that VEGF activity is a key feature during tumor growth and angiogenesis, and that blocking of this signal transduction pathway may inhibit tumor progression. *In vivo*, VEGF acts as a potent mitogenic factor for endothelial cells and as blood vessel permeabilising agents^[29]. Our *in vivo* study indicated that expression of VEGF was significantly reduced after As₂O₃ treatment, which demonstrated that As₂O₃ can inhibit VEGF expression. The formation of new blood vessels is inhibited due to suppression of tumor cell secretion of VEGF by As₂O₃.

We further explored the mechanism of the inhibitory action of As₂O₃ on VEGF in tumor cells *in vitro*. We measured expression of VEGF protein and VEGF mRNA in the SGC-7901 cell line with Western blots and real time RT-PCR separately. The results showed that As₂O₃ induced a dose- and time-dependent down-regulation of VEGF protein expression in the SGC-7901 cell line, but did not affect VEGF mRNA expression. The results reveal the effect of As₂O₃ on VEGF expression on the protein level rather than the mRNA level.

These studies have demonstrated that As₂O₃ can inhibit solid tumor growth *in vivo*, possibly by inhibiting the formation of new blood vessels. One of the mechanisms is that As₂O₃ can inhibit VEGF protein expression. These results may be useful in devising better strategies for cancer treatment.

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Prevalence of autoantibodies and the risk of autoimmune thyroid disease in children with chronic hepatitis C virus infection treated with interferon- α

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Abstract

AIM: To evaluate the prevalence of autoantibodies in chronic hepatitis C virus (HCV)-infected children focusing on thyroid autoimmunity.

METHODS: We investigated the prevalence of autoantibodies in 123 chronic HCV-infected children before, during and after monotherapy with interferon- α (IFN- α) or combined treatment with interferon- α or peginterferon- α and ribavirin. Besides antibodies against smooth muscle (SMA), nuclei (ANA), and liver/kidney microsomes (LKM), the incidence of anti-thyroid peroxidase antibodies as well as thyroid function parameters (TSH, FT3 and FT4) were determined.

RESULTS: We found that 8% of children had autoantibodies before treatment. During treatment, 18% of children were found positive for at least one autoantibody; 15.5% of children developed pathologic thyroid values during IFN- α treatment compared to only one child before therapy. Six children had to be substituted while developing laboratory signs of hypothyroidism.

CONCLUSION: Our data indicate a strong correlation between interferon- α treatment and autoimmune phenomena, notably the emergence of thyroid antibodies. The fact that some children required hormone replacement underlines the need of close monitoring in particularly those who respond to therapy and have to be treated for more than 6 mo.

INTRODUCTION

Since the discovery of the hepatitis C virus (HCV) in 1989^[1], an increasing number of studies report an association of chronic HCV infection with autoimmune phenomena. In studies among adult patients, the prevalence of autoantibodies in chronic HCV-infected patients varies from 25% to 66%^[2-5]. The most commonly detected autoantibodies are anti-smooth muscle antibodies (SMA), followed by anti-nuclear antibodies (ANA), and anti-liver/kidney microsomal antibodies (LKM). The latter have been associated with ALT flares during interferon- α (IFN- α) administration, leading to discontinuation of treatment^[6]. The non-organ-specific autoantibodies (NOSA) ANA, SMA and LKM detected during chronic HCV infection are also found in patients with autoimmune hepatitis (AIH). A discrimination of HCV-related appearance of autoantibodies and AIH is usually possible based on the detection of viral RNA, the subtype of the examined autoantibodies, histology and response to treatment^[4,7]. In rare occasions, an overlapping clinical and histological pattern of chronic HCV and AIH is found. In these cases, a careful assessment of either an immunosuppressive or anti-viral therapeutic approach is necessary^[8].

Various studies in adult patients addressed the question of whether the presence of NOSA in chronic HCV infection influences the response to IFN- α treatment. NOSA detection correlates with significantly higher ALT levels and higher histological activity^[3,4]. The majority of studies did not observe any effects of NOSA on the final outcome of treatment^[4-6]. The limitation of most of these

studies is that they only deal with IFN- α monotherapy. A very recent study investigating the effects of NOSA in patients receiving combined treatment not only detected a significantly higher viral load and higher ALT levels, but also observed a poorer response to treatment^[2]. Besides the detection of autoantibodies, the natural course of HCV infection is accompanied by an increased prevalence of autoimmune diseases^[9,10].

One of the most common phenomena in chronic HCV infection is the appearance of autoantibodies against the thyroid^[11-15]. A significant percentage of patients with anti-thyroid antibodies develop signs of thyroid dysfunction, predominantly hypothyroidism^[12]. Several studies observed that IFN- α treatment increased the risk of thyroid dysfunction^[13-15], whereas in most cases, thyroid dysfunction is reversed after discontinuation of IFN- α treatment. A recent study by Carella *et al*^[16] observed the development of long-term thyroiditis following IFN- α therapy. It is important to note that IFN- α treatment itself is associated with autoimmune manifestations^[17]. These are not only observed in the context of HCV treatment, but also when IFN- α is used as a therapeutic agent in various oncologic diseases^[18].

The prevalences of autoantibodies and risk to develop autoimmune diseases in children and adolescents with chronic HCV have only been investigated in three pediatric cohorts^[7,19-21]. These studies have in common that they: (1) analyzed intensively the overall prevalence of autoantibodies in HCV-infected children; (2) correlated autoantibody appearance with IFN- α monotherapy; and (3) investigated the response of LKM-positive children to IFN- α treatment. However, they did not elucidate the emergence of NOSA and thyroid autoantibodies under combination treatment.

Based on our experience with children requiring thyroid hormone replacement during treatment for chronic HCV, a systematic surveillance is needed in order to avoid harmful clinical side effects.

We therefore aimed to investigate the prevalence and dynamics of the appearance of autoantibodies in our cohort of HCV-positive children that underwent IFN- α treatment with and without ribavirin, to analyze the risk of chronically HCV-infected children to develop clinical or laboratory signs of thyroid dysfunction, and to obtain follow-up data after ceasing therapy.

MATERIALS AND METHODS

Patients

We evaluated a total of 123 children with chronic HCV infection who underwent IFN- α treatment. The diagnosis of chronic hepatitis C was based on: (1) presence of abnormal alanine aminotransferase (ALT) levels; (2) detection of anti-HCV antibodies; (3) detection of HCV-RNA over a period of at least 6 mo; and (4) histological evidence of hepatitis in patients with available liver biopsy. None of the patients had serological evidence of co-infection with hepatitis B, delta hepatitis, HIV, or clinical signs of an autoimmune disease prior to treatment with IFN- α . After interferon first became available for treatment of children, 21 children were treated with

Table 1 Epidemiological, clinical, and biochemical baseline data for HCV-infected patients who underwent either IFN- α monotherapy or combined therapy with IFN- α or peginterferon- α plus ribavirin

	IFN- α (<i>n</i> = 21)	IFN- α + ribavirin (<i>n</i> = 40)	Peginterferon- α + ribavirin (<i>n</i> = 62)
Male/female (<i>n</i>)	12/9	19/21	29/33
Median age, yr (range)	9.5 (2-17)	8.1 (2-16)	10.6 (2-17)
Route of infection, <i>n</i> (%)			
Parenteral	12 (57%)	14 (35%)	28 (45.1%)
Vertical	9 (43%)	21 (52.5%)	25 (40.3%)
Unknown	-	5 (12.5%)	9 (14.6%)
Median time between diagnosis and treatment, yr (range)	2.8 (1-9)	2.5 (1-5)	Not determined
Response to treatment, <i>n</i> (%)			
Sustained	6 (29%)	24 (60%)	36 (59%)
Transient	3 (14%)	1 (2.5%)	7 (11%)
No response	12 (57%)	15 (37.5%)	19 (30%)
Median ALT level (U/L) (range)			
Before treatment	39 (13-386)	35.5 (7-90)	41.6 (11-293)
After treatment (24 mo)	35 (9-137)	22.1 (6-104)	23.1 (7-71)

IFN- α alone. Additionally, 102 chronically HCV-infected children received combined treatment of IFN- α or peginterferon- α and ribavirin^[22,23]. Forty nine percent of the children were male. The predominant route of infection for the interferon-monotherapy treatment group was parenteral (57%), whereas, for the combined treatment group, parenteral and vertical infections were almost the same (41% *vs* 45%). The age ranged between 2-17 years at the beginning of treatment with a median age of approximately 9 years. The average time between diagnosis and start of treatment was 2.5 years (Table 1).

Treatment

In addition to those mentioned above, inclusion criteria for treatment of chronic HCV infection were normal values for hemoglobin, platelets, white blood cells, bilirubin, glucose, and serum creatinine. Criteria for exclusion were underlying systemic disease, metabolic liver disorders, prior immune suppressive therapy, and severe neurologic impairment. The parents of each patient gave written consent and the University Ethics Committee approved the studies.

Children treated with IFN- α received recombinant 5 mU IFN- α -2b/m² of body surface inoculated subcutaneously (sc) 3 times weekly over a period of 12 mo. Children treated with IFN- α combined with ribavirin received the same dose of IFN- α or 1.5 μ g/kg peginterferon- α -2b once a week and 15 mg/kg ribavirin twice daily orally over 12 mo. Patients who remained HCV-RNA seropositive 6 mo after the beginning of treatment discontinued therapy. Complete sustained virologic response was defined as normalization of serum aminotransferase levels and undetectable HCV RNA during the course of treatment and persisting during the entire post-therapy follow-up.

Screening for auto-antibodies and thyroid markers

Serum samples were taken at the time of primary

Table 2 Positive¹ autoantibodies in the two treatment groups before, during and 12 mo after treatment

Group	n	Before treatment			During treatment			After treatment		
		LKM ²	SMA ³	ANA ⁴	LKM ²	SMA ³	ANA ⁴	LKM ²	SMA ³	ANA ⁴
IFN-monotherapy	21	1	0	0	2	0	2	1	1	2
IFN /ribavirin-combined treatment	18	0	0	2	0	2	1	0	0	0
Total	39	1	0	2	2	2	3	1	1	2

¹Antibody titer was considered positive at values $\geq 1:40$; ²Liver kidney microsomal antibody; ³Anti-smooth muscle antibody; ⁴Antinuclear antibody.

diagnosis, before, during and after treatment. During treatment, samples were taken at 3 mo intervals. Antinuclear antibodies (ANA), anti-smooth-muscle antibodies (SMA), and antibodies against liver/kidney microsomes (LKM) were assessed by indirect immunofluorescence (IFL) on cryostat sections of rat liver and kidney specimens. ANA-positive samples were subsequently tested by IFL on Hep-2 cells. Antibody titers $\geq 1:40$ were considered positive. Along with testing auto-antibodies, thyroid function was evaluated by measuring the serum levels of free triiodothyronine (FT3; normal values: 1.8-4.6 ng/L), free thyroxine (FT4; normal values: 0.9-1.7 ng/dL) and thyroid-stimulating hormone (TSH; normal values: 0.3-4.2 mU/L). The sera were analyzed on site using commercially available kits. Furthermore, we determined anti-thyroglobulin antibody (TGA; normal values < 50 U/L) and anti-thyroid peroxidase antibodies (TPO; normal values: < 35 IU/mL) in the samples.

Statistical analysis

Results were analyzed using the SigmaStat 3.0 statistics program (Jandel Scientific, San Rael, CA). When comparing more than two groups, a one-way ANOVA was performed, followed by a Dunn's test to determine which groups differed significantly. $P < 0.05$ was considered statistically significant.

RESULTS

Duration of treatment

Of the 21 children treated with IFN- α -2b monotherapy, 12 (57%) remained HCV-RNA-positive and therefore discontinued after 6 mo, 3 patients showed a transient response with reappearance of viral RNA, and 6 (29%) children had a sustained response. Thus, 9 individuals were treated for 12 mo (Table 1). Forty children were enrolled in the second study that consisted of a combination treatment with IFN- α and ribavirin^[22]. Fifty seven percent of the treated children and adolescents displayed a transient or sustained virologic response and were treated for 12 mo. With introduction of peginterferon- α into the combined treatment group, the percentage of children with sustained response remained at a similar level. A total of 39 (63%) individuals were treated for 12 mo^[23].

Prevalence of non-organ specific autoantibodies

A total of 39 children had a complete record of their autoantibody status (Table 2). Before treatment, 3 of the 39 (8%) were positive for autoantibodies. One child was

positive for LKM and two children were positive for SMA. All measured antibody titers were in a low range between 1:40 and 1:80. None of the children had any apparent signs of autoimmune disease. The child found positive for LKM antibodies had slightly elevated ALT levels (56 U/L) and was closely monitored.

Autoantibodies and interferon treatment

During interferon treatment, there was a significantly higher number of autoantibody-positive children. Altogether, 7 of 39 (18%) children were positive for either LKM, SMA, or ANA. In all cases, the patients were positive for only one of the autoantibodies tested. The antibody titers were higher than that measured before treatment (maximum = 1:320). None of the children had any clinical signs of autoimmune disease, which would cause interferon treatment to be discontinued. Both children, who were LKM-positive, displayed stable ALT levels during treatment. When correlating ALT levels and autoantibody prevalence, we found slightly higher ALT values in autoantibody-positive children (average ALT level 54.5 U/L *vs* 40.9 U/L). Autoantibody prevalence did not correlate with treatment outcome.

Influence of interferon on thyroid function

None of the children with chronic HCV prior to treatment had clinical signs of thyroid dysfunction. Except for two children, all had normal values for TGA, TPO, TSH, FT3, and FT4 (Table 3). In the case of one child, TSH was slightly elevated (5.45 mU/L) without any apparent clinical sign or laboratory value of thyroid dysfunction. The other child had borderline thyroid antibody levels, with an otherwise normal laboratory and clinical evaluation. During treatment, 19 (15.5%) children had at least one pathologic thyroid parameter. When these children were further analyzed, significant increases in serum TPO and TSH levels were observed (Table 4). Of the 14 children that became positive for TPO antibodies, 12 also displayed increased serum TSH values. The increase in TSH was accompanied by a 50% decrease in serum FT3 and a slight decrease in FT4. Because of pathologic thyroid values, 6 children received a weight-adjusted dose of L-thyroxin until completion of treatment. The substitution could be discontinued in 4 cases during the follow-up period. Two children required hormone supplementation 12 mo after the cessation of the ribavirin/IFN- α treatment. Despite significant increases in pathologic thyroid values, the interferon-treated children did not reveal any clinical signs of hypo- or hyperthyroidism upon physical examination.

Table 3 Thyroid function tests in the two treatment groups before, during and 12 mo after treatment

Group	n	Before treatment				During treatment				After treatment			
		TGA ¹	TPOAb ²	TSH ³	FT3 ⁴ /FT4 ⁵	TGA ¹	TPOAb ²	TSH ³	FT3 ⁴ /FT4 ⁵	TGA ¹	TPOAb ²	TSH ³	FT3 ⁴ /FT4 ⁵
IFN-mono-therapy	21	0	0	1	0	3	5	3	0	0	0	0	0
IFN/ribavirin-combined treatment	40	0	0	0	0	5	2	3	1	1	0	0	0
Peginterferon- α + ribavirin	62	ND	1	0	ND	ND	7	6	ND	ND	3	0	ND
Total	123	0	1	1	0	8	14	12	1	1	3	0	0

¹Thyroglobulin antibody (normal range < 50 U/L); ²Thyroid peroxidase antibody (normal range < 35 IU/mL); ³Thyroid-stimulating hormone (normal range 0.3-4.2 mU/L); ⁴Free triiodothyronine (normal range: 1.8-4.6 ng/L); ⁵Free thyroxine (normal range 7-19 ng/L). ND = not determined.

Table 4 Overview of the thyroid function and auto-antibody titers of the 12 children who developed pathologic values during the course of IFN- α treatment Median (range)

Treatment	TGA ¹	TPOAb ²	TSH ³	FT3 ⁴	FT4 ⁵
Before	29.0 (9.4-53.0)	3.2 (0-41.0)	2.7 (1.0-5.5)	3.15 (2.1-5.3)	1.2 (1.1-1.3)
During	100.0 (8.0-141.0)	82.5 ^a (4-3 909)	7.3 ^a (1.7-76.0)	2.12 (1.1-4.3)	1 (0.4-1.3)
After	40.0 (13-163.0)	4.5 (0-289.0)	2.2 (0.9-4.2)	4.0 (2.0-5.3)	1.1 (1.0-1.3)

^a $P < 0.05$ vs before and after treatment. ¹Thyroglobulin antibody (normal range < 50 U/L); ²Thyroid peroxidase antibody (normal range < 35 IU/mL); ³Thyroid-stimulating hormone (normal range 0.3-4.2 mU/L); ⁴Free triiodothyronine (normal range 1.8-4.6 ng/L); ⁵Free thyroxine (normal range 7-19 ng/L).

Comparing the two treatment groups, there were more patients with TPO antibodies in the interferon monotherapy group (23%) than in the combined treatment group (9%). This difference was also apparent, but not as prominent, with regards to pathologic TSH values, which were 14% in the monotherapy group *versus* 10% in the group that underwent combined treatment. Notably, the prevalence of thyroid antibodies increased over the treatment period, particularly in the second half. While only 4.9% of children were positive after 3 mo, 12.8% were positive after 9 mo of medication.

DISCUSSION

In this study, we investigated the prevalence of autoimmune phenomena in chronic HCV-infected children treated in Germany. As we reported recently, over 50% of children and adolescents treated with IFN- α plus ribavirin displayed a sustained virologic response^[22,23]. In the present study, we also incorporated HCV-positive children treated solely with IFN- α before the implementation of combination therapy. Concordant with other reports, these children had a sustained response in 29% of all cases^[24]. Of the 39 children with complete autoantibody evaluation, 3 (2 ANA/1 LKM) were positive for autoantibodies before treatment. The overall prevalence of non-organ-specific autoantibodies (8%) was significantly lower than that found in the three published pediatric cohorts (32.5%-65%)^[7,19,21]. Surprisingly, none of our children was SMA-positive before IFN- α treatment, compared to 17%-67% of the children in the aforementioned studies^[7,19,21]. The number of autoantibody-positive children increased to 23% during treatment, which is still significantly lower than previously reported, then dropped to an approximate 10% after treatment completion. In contrast to the other pediatric cohorts, the two patients that were LKM-positive did not

develop any significant ALT flare during IFN- α treatment.

Two possible factors might explain the differences observed in autoantibody prevalence: (1) the methodology used and cut-off for positive results, and (2) geographic differences and, therefore, differences in genetic predisposition. Interestingly, all the published pediatric cohorts came from various regions in Italy; the majority of the patients in our cohort were from Germany or Eastern Europe. It is well known that autoimmune diseases are influenced by genetic background and HLA type^[25-27]. We compared liver enzyme levels in the sera of autoantibody-positive and -negative children. Average ALT levels were higher in the autoantibody-positive group, though the difference was not statistically significant. This finding correlates with similar observations in pediatric and adult cohorts^[4,7]. As in the majority of adult studies, we could not detect any connection between autoantibody appearance and treatment outcome^[4-6]. A very recent study in adults observed a better response in autoantibody-negative patients treated with IFN- α plus ribavirin^[2]. We did not observe a similar tendency in children that received combined treatment.

To the best of our knowledge, this is the first pediatric study that investigates the correlation between chronic HCV infection and thyroid dysfunction. Before initiation of treatment, all children were euthyroid and only one child had low levels of TPO antibodies. This finding differs significantly from most adult studies, which report 4.8%-21% TPO autoantibody prevalence in their patient cohorts and up to 13% of patients with thyroid dysfunction^[11,12,15]. A very recent publication by Mandac *et al*^[28] emphasizes again the clinical significance of thyroiditis in patients receiving interferon therapy and suggests the classification as autoimmune type and non-autoimmune type. During the course of IFN- α treatment, 11% of the children became positive for antibody

specific for TPO. The prevalence of TPO antibody was accompanied by a significant increase in TSH levels and a decrease in FT3 values. Six children became hypothyroid and had to be treated. The risk of developing signs of thyroid dysfunction became particularly prominent during the 2nd half of the treatment period. Overall, the number of HCV-infected children with thyroid dysfunction was significantly lower and strongly associated to IFN- α treatment, which is in contrast to adult studies that reported a high prevalence of thyroid dysfunctions even before IFN- α treatment.

In summary, we found a lower prevalence of non-organ-specific, as well as thyroid-specific autoantibodies than reported in related pediatric and adult cohorts. The prevalence of NOSA and thyroid antibodies strongly correlated with IFN- α treatment; six children developed signs of hypothyroidism. Since serum thyroid antibodies and TSH levels increased significantly in the second 6-mo treatment period, some individuals may require thyroid hormone substitution. It must be emphasized that close monitoring of thyroid function is mandatory, especially in children who respond to therapy and have to be treated for more than 6 mo.

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Human β -defensin-3 induction in *H pylori*-infected gastric mucosal tissues

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Abstract

AIM: To examine human β -defensin-3 (hBD-3) expression in inflamed gastric mucosal tissues or MKN45 gastric cancer cells with or without *H pylori* infection for better understanding the innate immune response to *H pylori*.

METHODS: We used reverse transcription-polymerase chain reactions and immunohistochemistry to examine hBD-3 expression in inflamed gastric mucosal tissues or MKN45 gastric cancer cells with or without *H pylori*. Effects of hBD-3 against *H pylori* were also evaluated.

RESULTS: The mean mRNA expression of hBD-3 in *H pylori*-positive specimens was significantly higher than that in *H pylori*-negative specimens ($P = 0.0002$, Mann-Whitney). In addition, unlike uninfected samples, 8 of 15 (53.33%) infected mucosal samples expressed hBD-3 protein. *H pylori* dose-dependently induced mRNA expression of hBD-3 in MKN45 cells, an effect inhibited by adding anti-toll-like receptor (TLR)-4 antibody. hBD-3 protein completely inhibited *H pylori* growth.

CONCLUSION: Our results suggest that like hBD-2, hBD-3 may be involved in the pathophysiology of *H pylori*-induced gastritis.

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Key words: Human β -defensin-2; Human β -defensin-3; *H pylori*; Gastric mucosa

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INTRODUCTION

Defensins are antimicrobial peptide components of the innate immune system. Three subfamilies, α -defensins, β -defensins, and θ -defensins, distinguished according to structural features at the gene and protein levels, have been identified in vertebrates^[1-3]. Four human β -defensins (hBD) have recently been characterized in various human epithelial cells. hBD-1 mRNA is expressed constitutively in various epithelial tissues^[1-2]. hBD-2 mRNA expression has been detected in epithelial cells of skin, lung, trachea, and urogenital tract; expression can be induced by treatment with tumor necrosis factor (TNF)- α or interleukin (IL)-1, or by exposure to microorganisms^[1-9]. Both hBD-1 and hBD-2 show antimicrobial activity, predominantly against Gram-negative bacteria. In skin, tonsil, and trachea, hBD-3 mRNA expression can be induced in epithelial cells by treatment with TNF- α or contact with *Pseudomonas aeruginosa*^[9]. hBD-3 protein shows antimicrobial activity against both Gram-negative and Gram-positive bacteria^[9]. Intense hBD-4 mRNA expression has been found in testis and gastric antrum, with lower, presumably constitutive, expression observed in uterus, thyroid gland, lung, kidney, and neutrophils^[10]. hBD-4 mRNA expression can be induced by treatment with phorbol 12-myristate 13-acetate (PMA) or contact with *P. aeruginosa* or *Streptococcus pneumoniae* in SAEC 6043 small airway epithelial cells, but not by IL-1, IL-6, interferon (IFN)- γ , or TNF- α . hBD-4 shows antimicrobial activity against both Gram-negative and Gram-positive bacteria^[10]. However, isolation of natural hBD-4 has not yet been reported.

Recently, induction of hBD-2 mRNA expression by *H pylori* has been shown in human gastric cancer cell lines (MKN 45 and AGS)^[4-8]. Gastric colonization by *H pylori*, which is Gram negative, is pathogenetically important in gastritis, peptic ulcer, gastric cancer, and gastric mucosa-associated lymphoid tissue lymphoma. However, hBD-3 mRNA expression in gastric mucosal tissues has not been fully characterized^[1].

To better understand the innate immune response to *H pylori*, we determined hBD-3 expression in various gastric mucosal tissues with or without *H pylori* infection using a semiquantitative TaqMan reverse transcription-

polymerase chain reaction (RT-PCR) assay as well as immunohistochemistry. Additionally, the antimicrobial effect of hBD-3 against *H. pylori* was evaluated.

MATERIALS AND METHODS

Bacterial strain and antibodies

H. pylori (ATCC49504) was used for hBD-2 mRNA and hBD-3 mRNA induction. Anti-toll-like receptor (TLR)-4 antibody (Clone: HTA125) and non-immune subclass-matched antibody (IgG_{2a}) were purchased from BD Biosciences Pharmingen. Polyclonal goat antibodies against hBD-2 and hBD-3 were purchased from Santa Cruz Biotechnology.

HBD-2 mRNA and hBD-3 mRNA induction in MKN45 gastric cancer cells

MKN-45 gastric cancer cells were cultured in RPMI 1640 medium (Bio Whittaker) supplemented with heat-inactivated fetal bovine serum (FBS) (JRH BIOSCIENCES) at 37°C in an humidified atmosphere containing 50 mL/L CO₂. Induction of hBD-2 mRNA and hBD-3 mRNA was carried out as described previously^[7,8]. Briefly, 10⁶ MKN45 cells were seeded into dishes 60 mm in diameter and incubated for 12 h. Culture medium was replaced with 2 mL of fresh RPMI 1640 medium without FBS. Bacterial suspensions (100 µL; 0 to 10⁹ CFU/mL in RPMI 1640 medium) were added to the dishes, and incubation was continued for various time periods.

Tissue samples

Samples of non-cancerous mucosa with or without chronic gastritis were obtained from 25 patients with previously untreated gastric cancer following surgery at Sapporo Medical University Hospital. Informed consent was obtained from all patients. After tissue removal, all samples were immediately frozen and fixed in 100 mL/L formalin.

Determination of *H. pylori* infection

Sections were Giemsa-stained, and the rapid urease test (CLO test, Tri-Med Specialties Inc) was performed with fresh samples taken from the prepyloric antrum, greater curvature of the corpus, and fundus^[12]. *H. pylori* infection was defined as positive when *H. pylori* was detected and/or the CLO test was positive.

Quantitative RT-PCR assays for hBD-2 mRNA and hBD-3 mRNA

ISOGEN (Nippon Gene) was used to extract total RNA from cells or tissues, and this extract was assayed for RNA with the GeneQuant DNA/RNA calculator (Amersham Pharmacia Biotech). For quantitative RT-PCR, fluorescent hybridization probes, TaqMan PCR Core Reagents kit with AmpliTaq Gold (Perkin-Elmer Applied Biosystems) were used with the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). Expression of hBD mRNA was quantified as previously described^[8,13]. Primers and TaqMan probe for hBD-2 mRNA were

as follows: 5'-TGGTGGTATAGGCGATCCTGTT-3' (forward) and 5'-GGAGACCACAGGTGCCAATTT-3' (reverse); 5'-CCATATGTCATCCAGTTCTT-3' (TaqMan probe). Primers and TaqMan probe for hBD-3 mRNA were as follows. 5'-AGTGACCAAGCACACCTTTTCA-3' (forward) and 5'-CCAAAAACAGGAAGAGCAAAGC-3' (reverse); 5'-TATGAGGATCCATTATCTTCTGTT-3' (TaqMan probe). Aliquots of 25 ng of total RNA from samples was used for one-step RT-PCR. Conditions of one-step RT-PCR were as follows: 30 min at 48°C (stage 1, reverse transcription), 10 min at 95°C (stage 2, RT inactivation and AmpliTaq Gold activation), and then 40 cycles of amplification for 15 s at 95°C and 1 min at 60°C (stage 3, PCR). Data were normalized as the ratio of hBD-2 optical densities relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and were represented as arbitrary units.

Immunostaining

Formalin-fixed, paraffin-embedded tissue sections were stained with polyclonal goat antibodies against hBD-2, hBD-3 or non-immune goat serum using an indirect immunoperoxidase technique.

Antimicrobial assay

To evaluate the antimicrobial effects of hBD-2 and hBD-3 on *H. pylori*, 25 µL of 4 × 10⁶ CFU/mL *H. pylori* strain (ATCC49504) was cultured on HP agar (Eikenkagaku) after a 1-h pre-incubation at 37°C in the presence or absence of hBD-2 protein and hBD-3 protein (Peptide Institute). To determine the CFU number, the pre-incubation mixture was immediately diluted 100-fold with culture medium, and samples were cultured in triplicate. Viable cells (CFU/mL) were counted after 3 d in culture at 37°C.

RESULTS

Induction of hBD-2 mRNA and hBD-3 mRNA expression in MKN45 cells and various gastric mucosal tissue specimens

To clarify the effects of *H. pylori* on hBD-2 mRNA and hBD-3 mRNA expression, according to TaqMan RT-PCR, MKN45 cells first were incubated for 0 to 72 h with *H. pylori*. hBD-2 mRNA and hBD-3 mRNA expression was detected in MKN45 cells 12 h after initiating incubation with *H. pylori*, and then increased gradually (Figure 1).

To determine a suitable number of *H. pylori* bacteria for induction of hBD-2 mRNA and hBD-3 mRNA expression, 100 µL of 0 to 10⁹ CFU/mL *H. pylori* was incubated with MKN45 cells for 48 h, representing multiple inoculum sizes. hBD-2 mRNA and hBD-3 mRNA expression was up-regulated in a manner dependent on bacterial number (Figure 2), an effect first detectable at 10⁷ CFU/mL.

To evaluate the effect of *H. pylori* colonization in gastric tissues on hBD-2 and hBD-3 expression, mucosal samples showing gastritis from 15 *H. pylori*-positive and 10 *H. pylori*-negative patients were assessed by TaqMan RT-PCR

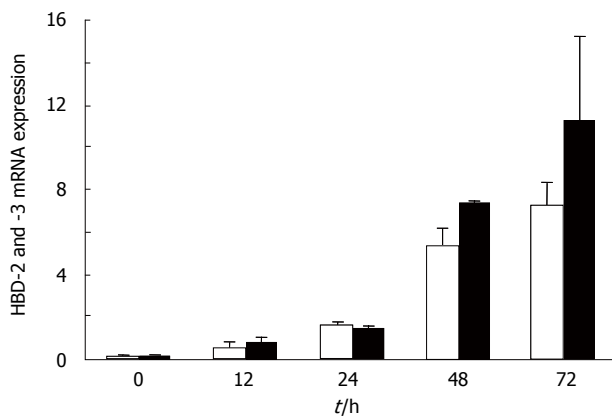


Figure 1 Time course of human β -defensin mRNA expression induced by *H. pylori* in a gastric cancer cell line MKN45. MKN45 cells (10^6) were seeded into dishes 60 mm in diameter and incubated for 12 h. Culture medium was replaced with 2 mL of fresh RPMI 1640 medium without FBS. The cells were incubated for 0 to 72 h with 100 μ L of 10^8 CFU/mL *H. pylori*. Human β -defensin mRNA expression was measured using TaqMan RT-PCR assay. hBD-2 (□), hBD-3 (■).

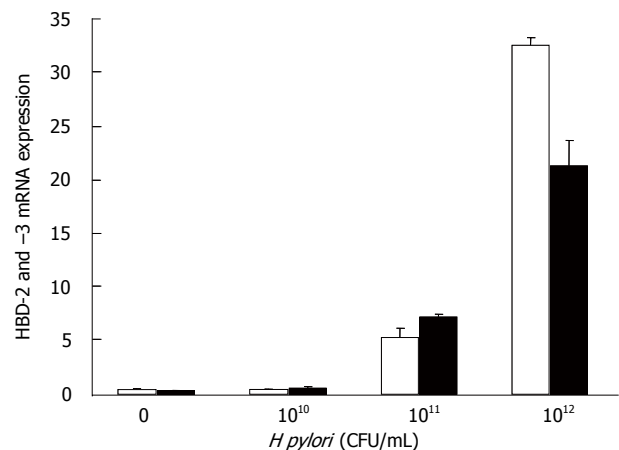


Figure 2 Induction of human β -defensin mRNA expression by various numbers of *H. pylori*. MKN45 cells (10^6) were seeded into dishes 60 mm in diameter and incubated for 12 h. Culture medium was replaced with 2 mL of fresh RPMI 1640 medium without FBS. Bacterial suspensions (100 μ L; 0 to 10^9 CFU/mL in RPMI 1640 medium) were added to the dishes, and incubation was continued for 48 h. Human β -defensin mRNA expression was measured using TaqMan RT-PCR assay. hBD-2 (□), hBD-3 (■).

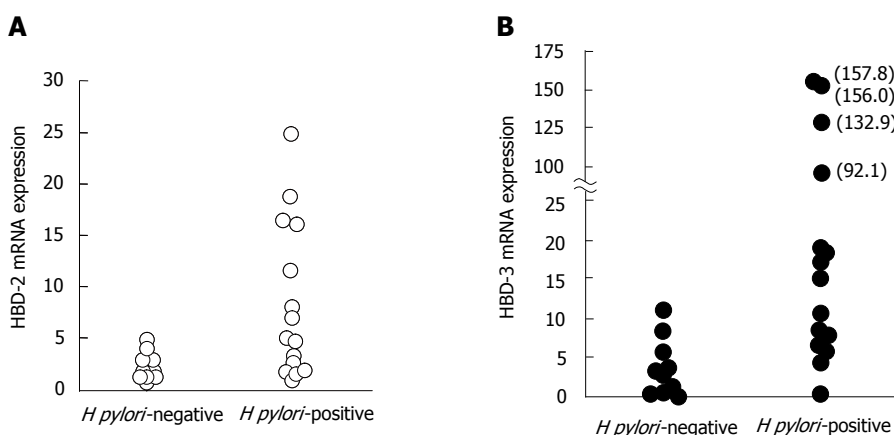


Figure 3 Human β -defensin mRNA expressions in gastric mucosal tissues from 15 *H. pylori*-positive patients (○) and 10 *H. pylori*-negative patients (●). Human β -defensin mRNA expression was measured using TaqMan RT-PCR assay. A: hBD-2; B: hBD-3.

analysis and immunostaining. Mean mRNA expression of hBD-2 (8.5) and hBD-3 (45.0) in *H. pylori*-positive specimens was significantly higher than that in hBD-2 (1.6) and hBD-3 (3.9) in *H. pylori*-negative specimens, respectively (Figure 3A and B; $P = 0.002$, Mann-Whitney). However, levels of hBD-3 mRNA expression did not correlate with those of hBD-2 mRNA expression in *H. pylori*-positive specimens. In addition, 7 and 8 of 15 *H. pylori*-positive patients with gastritis showed hBD-2 and hBD-3 protein expression in their mucosa, respectively, while none of the *H. pylori*-negative patients showed hBD-2 and hBD-3 protein expression (Figure 4A-D).

Inhibition of induction of hBD-2 mRNA and hBD-3 mRNA expression in MKN45 cells by treatment with anti-TLR-4 antibody

To determine whether hBD-2 and hBD-3 mRNA expression in MKN45 cells was induced via TLR-4, MKN45 cells were incubated for 40 h with 100 μ L of 10^9 CFU/mL of *H. pylori* in the presence or absence of anti-TLR-4 antibody (100 μ g/mL) and control antibody

(100 μ g/mL). Expression of hBD-2 and hBD-3 mRNA in *H. pylori*-exposed MKN45 cells treated with anti-TLR-4 antibody was reduced to 55.7% and 64.1% of the expression in untreated MKN45 cells, respectively.

Antimicrobial effects of hBD-2 and hBD-3 against *H. pylori*

To evaluate the antimicrobial effects of hBD-2 and hBD-3 on *H. pylori*, *H. pylori* was cultured on HP agar for 4 d after a 1-h pre-incubation in the presence or absence of chemically synthesized hBD-2 and hBD-3. At concentrations of 50 μ g/mL or more, hBD-2 and hBD-3 inhibited growth of *H. pylori* (Figure 5A and B).

DISCUSSION

Using TaqMan RT-PCR for hBD-3 mRNA and immunostaining for hBD-3 protein, we demonstrated, probably for the first time, that like hBD-2, hBD-3 is frequently expressed in gastric mucosa with *H. pylori* infection showing gastritis, but not in inflamed mucosa without *H. pylori* infection. The amount of hBD-3 mRNA

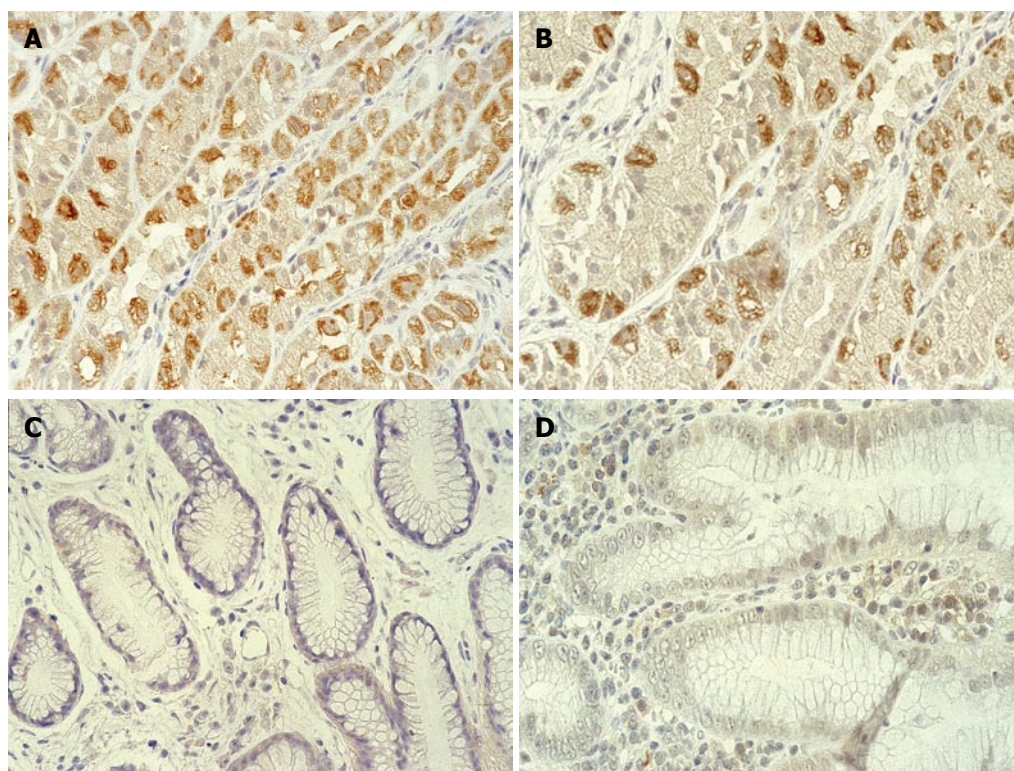


Figure 4 Human β -defensin protein expressions in gastric mucosal tissues with or without *H. pylori* infection. Tissues were stained with anti-hBD-2 antibody (A and C) or hBD-3 antibody (B and D). Case 1: A and B, gastric mucosa with *H. pylori*-associated gastritis. Immunostaining was observed in gastric cancer cells in A and gastric epithelial cells in B. Case 2: C and D, gastric mucosa with gastritis but without *H. pylori* infection. No staining was observed in C and D.

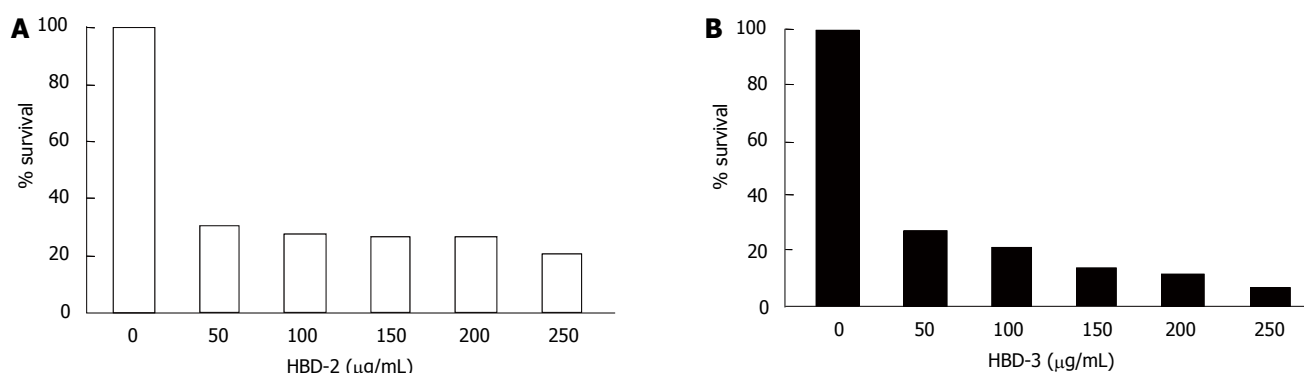


Figure 5 Antimicrobial effects of human β -defensin protein on *H. pylori* (ATCC49504). *H. pylori* were cultured on HP agar for 4 d after a 1-h pre-incubation in the presence or absence of hBD-2 (A) and hBD-3 (B).

expression in *H. pylori*-positive specimens was 11.5 times higher than in *H. pylori*-negative specimens. In addition, hBD-3 was induced by contact of MKN45 cells with *H. pylori*. Recent reports indicated that hBD-3 mRNA expression was induced in primary cultures of tracheal epithelial cells by pro-inflammatory cytokines, such as TNF- α , and in the MKN7 gastric cancer cell line by contact with *H. pylori*. Considered together with recent reports, our results implied that contact of gastric epithelial cells with *H. pylori* as well as amounts of pro-inflammatory cytokines are important for induction of hBD-3 mRNA expression.

At 10^{-5} mol/L, chemically synthesized hBD-2 (30 µg/mL) was reported to completely inhibit growth of *H. pylori*, while recombinant hBD-3 began to inhibit growth of *H. pylori* at a concentration of 10^{-7} mol/L^[5,11]. In the present study, we demonstrated that synthesized hBD-3 as well as hBD-2 inhibited *H. pylori* growth at concentrations of 50

µg/mL or more.

Isomoto *et al*^[14] detected activated nuclear factor (NF)- κ B in epithelial cells in gastric mucosa from patients with *H. pylori*-associated gastritis. Diamond *et al*^[15] reported that expression of hBD-3 was regulated by NF- κ B-independent mechanisms, that remain to be characterized. Su *et al*^[16] reported that *H. pylori* up-regulated TLR-4 expression in two gastric cancer cell lines (AGS and MKN45). These reports suggest that *H. pylori* may induce hBD-3 mRNA expression via direct or indirect activation of NF- κ B or other mechanisms. Pathogen-associated molecular patterns in *H. pylori* and pattern recognition receptors in gastric epithelial cells will require further study. In the present investigation, anti-TLR-4 antibody inhibited, but not completely, the expression of hBD-3 mRNA in MKN45 cells induced by contact with *H. pylori*. This suggests that not only *H. pylori* binding to TLR-4, but also other mechanisms may exist for the induction

of hBD-3 mRNA in gastric epithelial cells by *H. pylori* infection. In addition, in the present study, levels of hBD-3 mRNA expression did not correlate with those of hBD-2 mRNA expression in *H. pylori*-positive specimens. This also suggests that the expression of hBD-3 and hBD-2 mRNA in gastric epithelial cells is likely to be regulated differently.

In conclusion, expression of hBD-3 increases in response to *H. pylori* infection with a bacterial effect. Further study is needed to detect the role and regulating mechanism of hBD-3.

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BASIC RESEARCH

Bromophenacyl bromide, a phospholipase A₂ inhibitor attenuates chemically induced gastroduodenal ulcers in rats

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Abstract

AIM: To study the effect of bromophenacyl bromide (BPB), a phospholipase A₂ inhibitor on gastric secretion and to protect chemically induced gastric and duodenal ulcers in rats.

METHODS: Acid secretion studies were undertaken in pylorus-ligated rats with BPB treatment (0, 5, 15 and 45 mg/kg). Gastric and duodenal lesions in the rats were induced by ethanol and cysteamine respectively. The levels of gastric wall mucus, nonprotein sulfhydryls (NP-SH) and myeloperoxidase (MPO) were also measured in the glandular stomach of rats following ethanol induced gastric lesions.

RESULTS: BPB produced a dose-dependent inhibition of gastric acid secretion and acidity in rats. Pretreatment with BPB significantly attenuated the formation of ethanol induced gastric lesion. BPB also protected intestinal mucosa against cysteamine-induced duodenal ulcers. The antiulcer activity of BPB was associated with significant inhibition of ethanol-induced depletion of gastric wall mucus, NP-SH and MPO. These findings pointed towards the mediation of sulfhydryls in BPB induced gastrointestinal cytoprotection.

CONCLUSION: BPB possesses significant antiulcer and cytoprotective activity against experimentally induced gastroduodenal lesions.

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Key words: Bromophenacyl bromide; Phospholipase A₂; Gastric secretion; Gastric ulcer; Duodenal ulcer; Sulfhy-

dryls; Myeloperoxidase

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INTRODUCTION

Phospholipids play an important role in the preservation of gastrointestinal homeostasis^[1]. The gastric mucosa has a hydrophobic lining which is assumed to have protective functions against luminal acid as well as intrinsic and extrinsic corrosive agents^[2,3]. The hydrophobicity of the mucosal lining is attributed to a surfactant like phospholipid monolayer adsorbed to the mucosal surface which acts as a mucosal barrier and impedes back-diffusion of luminal H⁺ into the mucosal tissue and defends gastric mucosa against damage induced by strong acids^[4] and other barrier breaking agents^[5,6]. Altered phospholipids profile of gastric mucosa has been noticed in clinical gastropathies including *H. pylori* induced gastritis and peptic ulcers^[7,8]. Injury of the intestinal surface layer leads to an inflammatory reaction that is characterized by a variety of inflammatory mediators, activation of complement cascade system and of lipid mediator synthesis^[9]. In addition, damage of the gastrointestinal surface protection system and the breakdown of complex membrane lipids activate phospholipase A₂ (PLA₂), a key enzyme in the production of inflammatory lipid mediators^[7]. High concentrations of PLA₂ have been reported in gastric mucosa^[10,11]. PLA₂ mediated hydrolysis of membrane lipids results in membrane perturbation, cell degranulation and stripping of cell surface receptors^[10,12]. Intracellular PLA₂ plays an important role in inflammation by acting upon the cell membrane to release arachidonic acid from membrane phospholipids for the synthesis of eicosanoids, lypophospholipids and platelet activating factor^[13,14]. Arachidonic acid serves as a primary substrate for eicosanoid production yielding prostaglandins, leukotrienes and lipoxins; all of them have multiple vasoactive and cellular regulatory functions^[11,12]. There is abundant evidence suggesting the role of mucosal microcirculatory disturbances in experimental gastrointestinal damage^[15-17].

Exposure of gastric mucosa to necrotizing agents results in edema, vacuolization and necrosis of the luminal epithelial cells; these lesions morphologically resemble those caused by ischemic-reperfusion injury in the intestine^[18]. The ischemia is known to deplete tissue adenosine triphosphate (ATP) and significantly increase its metabolic product hypoxanthine, which in the presence of xanthine oxidase results in the production of xanthine and highly reactive superoxide radicals^[19]. Ischemic insult to intestinal mucosa causes excessive production of oxygen derived free radicals (ODFR) that may initiate a chain of reactions in membrane-bound lipids leading to lipid peroxidation and PLA₂ activation^[20,21]. Inhibition of this cascade by scavenging ODFR and/or inhibiting PLA₂ could be an effective tool to protect gastrointestinal mucosa against chemically induced lesions. Earlier studies have shown the protective effects of quinacrine, a PLA₂ inhibitor, against ischemia and injury of various organs including heart^[22], stomach^[23], intestine^[20] and lungs^[24]. Bromophenacyl bromide (BPB) is a PLA₂ inhibitor which is structurally different from quinacrine and inhibits PLA₂ by covalently binding to its active site rather than affecting the enzyme-substrate interface (mode of quinacrine-induced PLA₂ inhibition). The present investigation was aimed to study the effect of BPB on chemically induced gastroduodenal ulcers in rats.

MATERIALS AND METHODS

Animals and dosing

Albino Wistar rats of either sex, approximately of the same age, weighing 150 to 200 g and fed on standard chow diet were used. They were randomly divided into experimental groups of 6 rats each. The aqueous solutions of ulcerogens and BPB were freshly prepared before administration. BPB at doses of 5, 15 and 45 mg/kg was given intraperitoneally for gastric secretion studies and by gavage for antiulcer studies. The rats were sacrificed, and the stomachs removed and opened along the greater curvature. After washing with saline the gastric lesions were quantified by a person unaware of the treatments. The protocol of animal studies was approved by the Research and Ethics Committee of Armed Forces Hospital, Riyadh, Saudi Arabia.

Pylorus ligated rats

The rats were fasted for 36 h with access to water *ad libitum* before the pylorus was ligated under ether anesthesia, care being taken not to cause bleeding or to occlude blood vessels^[25]. BPB was administered immediately after pylorus ligation (Shay) by ip route. The rats were sacrificed at 6 h after the pylorus ligation. The stomachs were removed; the contents were collected, volumes measured, centrifuged and analyzed for titratable acidity against 0.01 mol/L NaOH to pH 7 and the total acid output was calculated.

Cysteamine-induced duodenal ulcers

Duodenal ulcers were induced by two doses of cysteamine hydrochloride (400 mg/kg ig in 10% aqueous solution) at an interval of 4 h according to the method described by

Szabo^[26]. BPB was administered 30 min before each dose of cysteamine. All the rats were sacrificed 24 h after the first dose of cysteamine and the duodenum was excised carefully and opened along the antimesenteric side. The duodenal ulcers were scored using a scale of 0 to 3, where 0 = no ulcer, 1 = superficial mucosal erosion, 2 = deep ulcer or transmural necrosis, and 3 = perforated or penetrated ulcer (into the pancreas or liver). The sum of the intensity of each lesion was used as the ulcer index.

Ethanol-induced gastric ulcers

The rats were administered (ig) with 1 mL of absolute ethanol^[27]. BPB was given 30 min before the administration of ethanol. One hour after the administration of ethanol the rats were sacrificed and examined for lesions in the stomach. The scoring of lesions, assays of gastric wall mucus and sulfhydryls in the stomach were done as follows: The patchal lesions of stomach induced by ethanol were scored according to the method described by Schiantarelli *et al.*^[28] using the following scale: 0 = normal mucosa; 1 = hyperemic mucosa or up to 3 small patches; 2 = from 4 to 10 small patches; 3 = more than 10 small or up to 3 medium-sized patches; 4 = from 4 to 6 medium-sized patches; 5 = more than 6 medium-sized or up to 3 large patches; 6 = from 4 to 6 large patches; 7 = from 7 to 10 large patches; 8 = more than 10 large patches or extensive necrotic zones. "Small" was defined as up to 2 mm across (max. diameter), "medium-sized" as between 2 and 4 mm across and "large" as more than 4 mm across.

Determination of gastric wall mucus

Gastric wall mucus was determined according to the modified procedure of Corne *et al.*^[29]. The glandular segment of the stomach was separated from the lumen of the stomach, weighed, and transferred immediately to 10 mL of 0.1% w/v Alcian blue solution (in 0.16 mmol/L sucrose solution buffered with 0.05 mL sodium acetate at pH 5). Tissue was stained for 2 h in Alcian blue, and excess dye was removed by two successive rinses with 10 mL of 0.25 mmol/L sucrose, first for 15 min and then for 45 min. Dye complexed with the gastric wall mucus was extracted with 10 mL of 0.5 mmol/L magnesium chloride which was intermittently shaken for 1 min at 30 min intervals for 2 h. Four milliliters of blue extract were then vigorously shaken with an equal volume of diethyl ether. The resulting emulsion was centrifuged at 4000 r/min for 10 min and the absorbance of aqueous layer was recorded at 580 nm. The quantity of Alcian blue extracted per gram of wet glandular tissue was then calculated.

Estimation of nonprotein sulfhydryls

Gastric mucosal nonprotein sulfhydryls (NP-SH) was measured according to the method of Sedlak and Lindsay^[30]. The glandular part of the stomach was homogenized in ice-cold 0.02 mmol/L EDTA. Aliquots of 5 mL of the homogenates were mixed in 15 mL test tubes with 4 mL of distilled water and 1 mL of 50% trichloroacetic acid. The tubes were shaken intermittently for 10 min and centrifuged at 3000 g. Two milliliters of supernatant were mixed with 4 mL of 0.4 mol/L Tris buffer at pH 8.9; 0.1

mL of DTNB [5, 5'-dithio-bis-(2-nitrobenzoic acid)] was added and the sample was shaken. The absorbance was measured within 5 min of addition of DTNB at 412 nm against a reagent blank.

Determination of myeloperoxidase activity

Myeloperoxidase (MPO) activity in the gastric mucosa was measured according to the methods described earlier^[31]. Pre-weighed tissue was homogenized (1:10 wt/vol) in 0.5% hexadecyltrimethyl ammonium bromide in 50 mmol potassium phosphate buffer (pH 6.0) before sonication in an ice bath for 20 s. Three freeze/thaw cycles were performed followed by sonication (20 s in ice bath). The samples were centrifuged at 17000 *g* (5 min, 4°C) and MPO in the supernatant was assayed by mixing of 0.1 mL of supernatant with 2.9 mL of 50 mmol/L potassium phosphate buffer (pH 6.0) containing 0.167 g/L o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured for 4 min using an UV-visible spectrophotometer (UV-160A, Shimadzu, Japan).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Differences with $P < 0.05$ were considered as statistically significant.

RESULTS

Effect of BPB on gastric secretions in 6 h pylorus-ligated rats

In control rats, pylorus ligation for 6 h resulted in the accumulation of 9.8 ± 0.55 mL of gastric secretions and a total acid output of 663 ± 48.7 mEq (Table 1). The volume of gastric secretion in the rats treated with 5, 15 and 45 mg/kg of BPB significantly reduced to 7.4 ± 0.42 mL, 6.6 ± 0.59 mL and 4.5 ± 0.47 mL respectively (ANOVA, $F = 17.25$, $P < 0.001$). A significant decrease in total acid output was observed in the rats treated with 15 mg/kg (193 ± 51.5 mEq) and 45 mg/kg (162 ± 34.8 mEq) of BPB (ANOVA, $F = 26.71$, $P < 0.001$). The rats treated with 5 mg/kg of BPB failed to show any significant change (576 ± 66.9 mEq) in total acid output as compared to control group (Table 1).

Effect of BPB on cysteamine-induced duodenal ulcers

Administration of cysteamine produced elongated lesions extending longitudinally down the duodenum. The lesion area of the rats in control group was 33.3 ± 0.21 mm². Treatment of rats with BPB reduced the area of lesions in all the groups. A significant reduction in lesion area was observed in the rats treated with 15 mg/kg (1.0 ± 0.26 mm²) and 45 mg/kg (1.5 ± 0.22 mm²) of BPB (ANOVA, $F = 17.45$, $P < 0.001$). The decrease in lesion area of the rats treated with 5 mg/kg (3.0 ± 0.36 mm²) was not statistically significant (Figure 1).

Effect of BPB on ethanol-induced gastric lesions

The treatment of rats with absolute ethanol produced extensive gastric lesions in the glandular mucosa of the

Table 1 Effect of BPB on gastric secretion and acidity in 6 h pylorus ligated rats (mean \pm SE)

Treatment	Gastric secretion (mL)	Total acid output (mmol)
Control (Ligation only)	9.8 ± 0.55	663 ± 48.7
Ligation + BPB 5 mg/kg	7.4 ± 0.42^b	576 ± 66.9
Ligation + BPB 15 mg/kg	6.6 ± 0.59^b	193 ± 51.5^b
Ligation + BPB 45 mg/kg	4.5 ± 0.47^b	162 ± 34.8^b

^b $P < 0.01$ vs control (Dunnett's test).

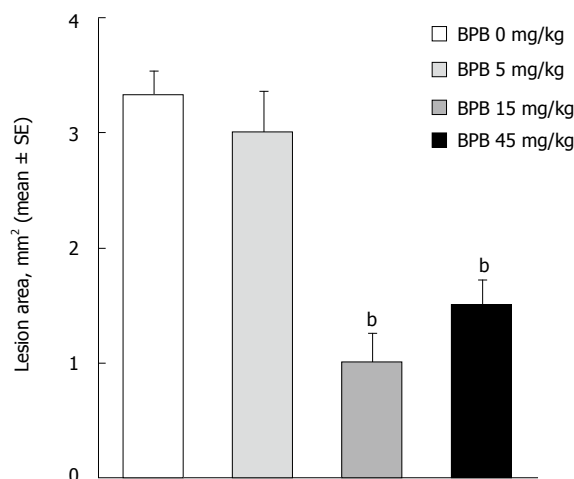


Figure 1 Effect of BPB on cysteamine-induced duodenal ulcers in rats. ^b $P < 0.001$ vs BPB 0 mg/kg (cysteamine only) (Dunnett's test).

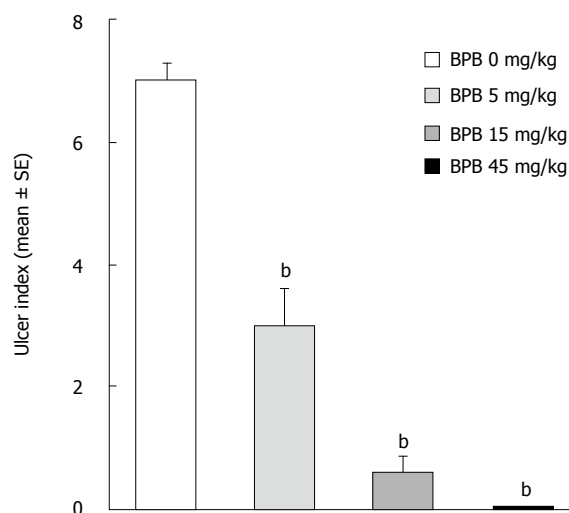


Figure 2 Effect of BPB on gastric mucosal damage induced by ethanol in rats. ^b $P < 0.01$ vs BPB 0 mg/kg (ethanol only) (Dunnett's test).

stomach in all the control rats. The ulcer index was 8.0 ± 0.31 in control rats 1 h after ethanol administration. Pretreatment of rats with BPB at the doses of 5 mg/kg (ulcer index = 3.0 ± 0.63), 15 mg/kg (0.6 ± 0.24) and 45 mg/kg (0.0 ± 0.0) significantly inhibited the formation of gastric lesions (ANOVA, $F = 72.07$, $P < 0.001$) (Figure 2). Ethanol-induced lesions were characterized by multiple

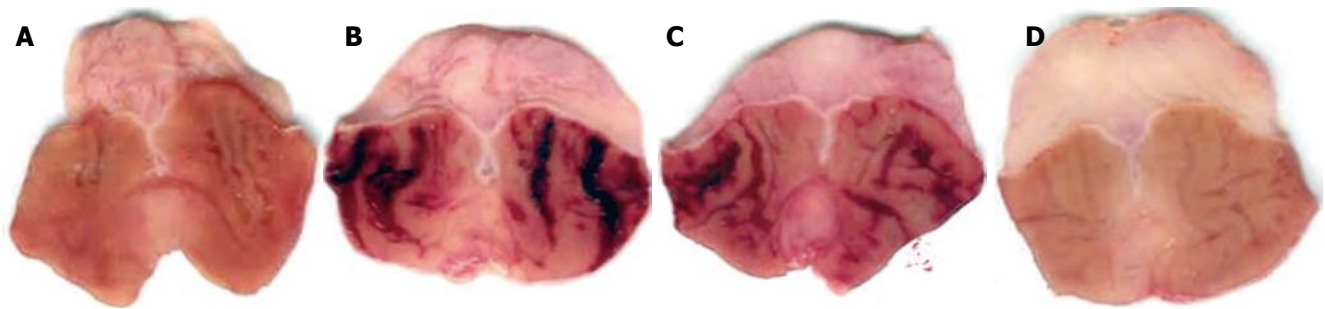


Figure 3 Morphological appearance of ethanol-induced band like hemorrhagic lesions in the stomach of rats. **A:** Normal mucosa; **B:** Ethanol produced lesion; **C:** Pretreatment of rats with BPB 15 mg/kg; **D:** Pretreatment of rats with BPB 45 mg/kg.

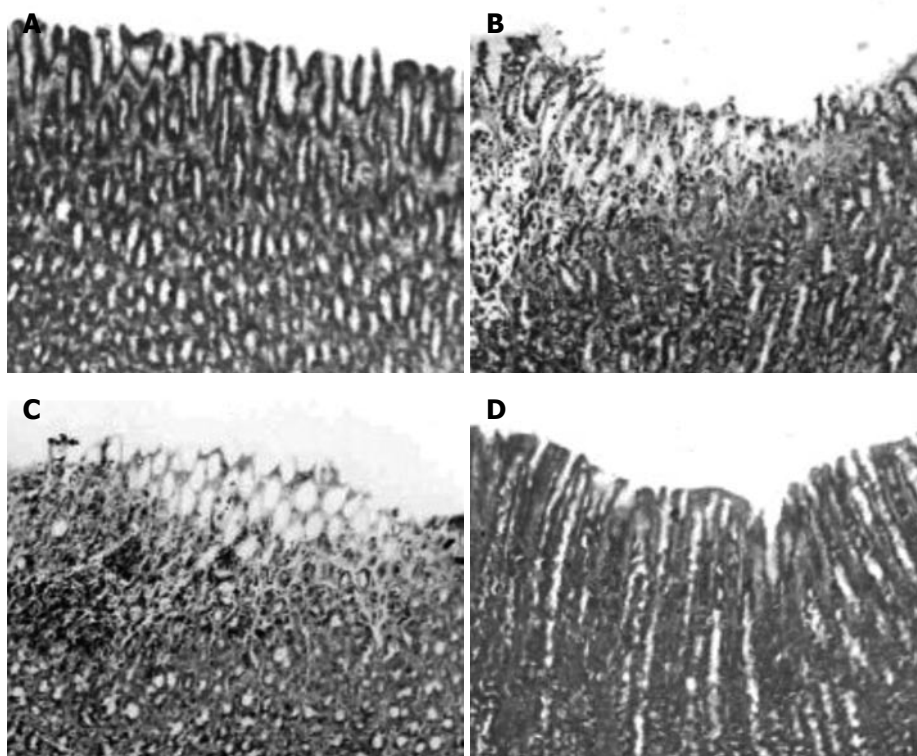


Figure 4 Light micrographs showing the effect of BPB on ethanol-induced gastric lesions of rats. **A:** Normal mucosa; **B:** Ethanol produced lesion; **C:** Pretreatment of rats with BPB 15 mg/kg; **D:** Pretreatment of rats with BPB 45 mg/kg.

hemorrhagic red bands (patches) of different size along the axis of the glandular stomach (Figure 3). Histological examination of gastric mucosa showed the appearance of these lesions in the form of gastric pits with detachment of the surface epithelium; epithelial cells appeared to be vacuolated and microvessels elongated (Figure 4). Pretreatment with BPB dose-dependently prevented ethanol-induced mucosal damage.

Effect of BPB on ethanol-induced changes in gastric wall mucus

The treatment of rats with ethanol significantly decreased the Alcian blue binding capacity of gastric wall mucus ($688 \pm 25.2 \mu\text{g}$ Alcian blue/g of tissue) as compared to control rats ($935 \pm 60.5 \mu\text{g/g}$). Pretreatment of rats with BPB at the doses of 5 mg/kg ($847 \pm 75.8 \mu\text{g/g}$), 15 mg/kg ($820 \pm 70.6 \mu\text{g/g}$) and 45 mg/kg ($927 \pm 34.2 \mu\text{g/g}$) significantly enhanced Alcian blue binding capacity of gastric mucosa (ANOVA $F = 3.09$, $P < 0.05$, Table 2).

Effect of BPB on ethanol-induced depletion of gastric mucosal NP-SH

The level of NP-SH in the gastric mucosa of control rats was $4.26 \pm 0.11 \mu\text{mol/g}$ of tissue, which was significantly decreased to $2.96 \pm 0.22 \mu\text{mol/g}$ following the administration of ethanol. Pretreatment of rats with BPB at all the three doses significantly inhibited ethanol-induced depletion of NP-SH (ANOVA $F = 13.76$, $P < 0.001$, Table 2).

Effect of BPB on ethanol-induced changes in gastric MPO activity

Changes in gastric accumulation of leukocytes following ethanol-induced lesions were evaluated by measurement of gastric MPO activity, which was significantly increased as compared to control mucosa (Table 2). Pretreatment with BPB significantly attenuated ethanol-induced increase in gastric MPO activity in rats (ANOVA, $F = 8.16$, $P < 0.001$).

Table 2 Effect of BPB on ethanol induced changes in Alcian blue binding capacity, NP-SH levels and MPO activity in gastric mucosa of rats (mean \pm SE)

Treatment	Alcian blue binding (mg/g tissue)	Non-protein sulfhydryl (mmol/g tissue)	Myeloperoxidase activity (Δ A/g tissue)
Normal	935 \pm 60.5	4.26 \pm 0.11	0.081 \pm 0.019
Ethanol (EtOH) alone	688 \pm 25.2 ^a	2.96 \pm 0.22 ^b	0.974 \pm 0.067 ^b
EtOH + BPB 5 mg/kg	847 \pm 75.8	4.08 \pm 0.12 ^d	0.123 \pm 0.024 ^d
EtOH + BPB 15 mg/kg	820 \pm 70.6	4.02 \pm 0.24 ^d	0.186 \pm 0.089 ^d
EtOH + BPB 45 mg/kg	927 \pm 34.2 ^c	4.80 \pm 0.19 ^d	0.067 \pm 0.025 ^d

^a $P < 0.05$ and ^b $P < 0.01$ vs control group; ^c $P < 0.05$, ^d $P < 0.01$ vs EtOH alone (Dunnett's test).

DISCUSSION

Pretreatment with BPB produced a dose dependent decrease in the volume and acid output of gastric secretions in Shay rats (Table 1). The increase in gastric acidity is considered as an important contributing factor in the pathogenesis of gastric and duodenal ulcers and is often termed as the 'aggressive factor'^[32]. The regulation of gastric acid secretion is complex and maintained by endogenous gastrin, histamine, somatostatin and cholinergic mechanisms^[33]. For acid secretion the expulsion of H⁺ across the epical membrane is coupled with the movement of K⁺ into the cell^[33]. Phospholipase A₂ inhibitors are known to modulate proton conductance across cell membranes^[34,35]. BPB has been shown to inhibit PLA₂ mediated histamine release from parietal cells in rats^[36]. A significant protective effect of BPB was observed against cysteamine-induced duodenal ulcers (Figure 1). The pathogenesis of cysteamine-induced duodenal lesions is far from clear. Cysteamine ulcers are considered to be associated with the hypersecretion of gastrin and hydrochloric acid and decreased mucosal resistance^[37]. The antiulcer activity of BPB may to some extent be attributed to its ability to inhibit gastric acid secretions (Table 1) and to preserve mucosal integrity (Table 2). Our results revealed that BPB significantly protected gastric mucosa against the depletion of gastric wall mucus (Table 2). The mucus gel adhering to the gastric mucosal surface protects the underlying epithelium against acid^[38,39], pepsin^[38] and necrotizing agents such as ethanol and indomethacin^[40]. It plays a more important role in the defense of the gastric mucosa against chemical or mechanical aggressions than the soluble mucus found in the lumen of the stomach^[41]. The gastric mucus coat is thought to be important in facilitating the repair of the damaged gastric epithelium^[42]. It seemed likely that the cytoprotective activity of this BPB could result, at least in part, from its interaction with the adhering gastric mucus layer.

Our results revealed that pretreatment of rats with BPB protected them against ethanol-induced gastric lesions (Figures 2-4). Numerous studies have indicated a substantial role for ODFR and PLA₂ in mediating ethanol-induced intestinal mucosal injury^[43,44]. The enhanced PLA₂ activity results in breakdown of membrane phospholipids

and activation of arachidonic acid cascade generating leukotrienes, prostaglandins and lipoxins^[45]. Prostacyclin is the major vasodilator and inhibitor of platelet aggregation, whereas thromboxan A₂ has the opposite effects^[46]. The ability of BPB to inhibit this proinflammatory cascade^[47-49] might be responsible for protecting gastric mucosa against chemically-induced lesions. The cytoprotective effect of BPB was accompanied by attenuation of ethanol-induced increase in MPO, a marker of neutrophil activity (Table 2). Neutrophils are the major inflammatory cell type infiltrating the injured mucosa following exposure to ethanol^[50]. Strategies to counteract neutrophil infiltration/activation have been shown to protect animals against gastric ulcers^[51,52].

A significant decrease in gastric NP-SH following ethanol administration indicated massive generation of ODFR (Table 2). Our findings are in agreement with earlier reports showing depletion of sulfhydryls in ethanol-induced gastric lesions^[53,54]. Within an inflammatory process, ODFRs are generated and initiate a chain reaction within membrane-bound lipids leading to lipid peroxidation. Treatment of rats with glutathione depletors has been shown to significantly potentiate ulcerogen-induced gastric mucosal injury^[55], whereas increase in mucosal NP-SH exerts gastroprotective effect^[56,57]. Sulfhydryl compounds play an important role in the formation of gastrointestinal mucus, which protects underlying gastric mucosa against necrotizing agents^[58,59]. Moreover, sulfhydryls have the ability to scavenge ODFR produced in tissues following exposure to cytotoxic compounds^[60]. These observations clearly point towards the mediation of sulfhydryls in BPB induced gastroprotection.

In conclusion, our findings show that BPB possesses both antisecretory and antiulcer effects. Further studies are required to determine the role of BPB in the prophylaxis and/or the treatment of gastrointestinal ulcer diseases.

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Effect of intestinal lymphatic circulation blockage in two-hit rats

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CONCLUSION: Ligation of mesenteric lymph duct could improve the disturbance of organic function and morphologic damage in two-hit rats; the lymphatic mechanism in two-hit should be emphasized.

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Key words: Two-hit; Mesenteric lymph duct; Ligation; Organs; Humoral factor

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Abstract

AIM: To study the effect of blocking intestinal lymphatic circulation in two-hit rats and explore the significance of intestinal lymphatic circulation in two-hit.

METHODS: Wistar rats were divided equally into three groups: mesenteric lymph duct ligation group, non-ligation group and sham group. Mesenteric lymph was diverted by ligation of mesenteric lymph duct, and the two-hit model was established by hemorrhage and lipopolysaccharide (LPS) methods. All rats were sampled for serum pre-experiment and 24 h post-experiment. The organs including kidney, liver, lung and heart were collected for pathomorphologic observation and biochemical investigation. The nitric oxide (NO), malondialdehyde (MDA) and superoxide dismutase (SOD) were determined in serum and tissue homogenate.

RESULTS: Pathomorphology study showed that the structures of kidney, lung, liver and heart tissues were normal in sham group; congestion, degeneration and necrosis in non-ligation group; but only mild lesions in ligation group. After two-hits, the contents of AST, ALT, BUN, Cr and LDH-1 in the serum of non-ligation group and ligation group were obviously higher than that in pre-experiment group and sham group, but obviously lower than that in non-ligation group. The contents of NO₂⁻/NO₃⁻, NOS, iNOS and MDA in the serum of non-ligation group were significantly increased, compared with pre-experiment and sham group, but SOD was significantly lower. These parameters were significantly different in ligation group compared with that in sham group, but NO₂⁻/NO₃⁻, iNOS and MDA in ligation group were significantly lower than that in non-ligation group.

INTRODUCTION

Multiple organ dysfunction syndrome (MODS) is a common but poorly understood complication in a variety of critical illnesses^[1-3]. In recent years, much attention has been paid to the study of MODS, and encouraging progress has been achieved. It has been accepted that the lymph system has an independent function and significance, which is different from the traditional concept that it is an assistant system of organic fluid circumfluence. In various pathogenesis of MODS, the complicated net of inflammatory mediators and cytokines is increasingly noticed. It has always been believed that their transport and transmit are *via* blood^[4-6]. However, whether the lymphatic pathway is meaningful to pathogenesis of MODS is yet to be clarified. We previously showed that lymph microcirculation is closely related to the occurrence and development of shock^[7,8]. Deitch *et al*^[9-12] reported that mesenteric lymph could activate neutrophils *in vivo* and *ex vivo*, promote the development of acute lung injury after resuscitation of hemorrhagic shock; when mesenteric lymph duct was ligated in advance, lung injury could be avoided. In the present study, in order to disclose the pathogenic importance of mesenteric lymph in MODS, the two-hit model was duplicated by two-hits of hemorrhage/resuscitation and lipopolysaccharide (LPS); the protective effect of intestinal lymphatic circulation block on organs in two-hit rats was investigated, and the influence of mesenteric lymph duct ligation on nitric oxide (NO) and free radicals in two-hit rats was explored.

MATERIALS AND METHODS

Animals

Forty-five male Wistar rats weighing 280 to 350 g (supplied by the Experimental Animal Center of Hebei Medical University), were divided equally into three groups: mesenteric lymph duct ligation group, non-ligation group and sham group.

Duplication of two-hit model

Rats were generally anesthetized with pentobarbital sodium (50 mg/kg) by intramuscular injection. A median incision was made in nuchae. The right common carotid arteries and left jugular veins were dissected, and cannulated to facilitate the blood withdrawal and infusion resuscitation. The two-hit model was duplicated by modified Spain's method^[13]. Blood (one sixth of body blood volume and one thirteenth of avoirdupois) was drawn by the automatic withdrawal-infusion machine (type of ZCZ-50) until the mean arterial pressure fell to a low level (less than 50 mmHg). The rate in the beginning was 0.4 mL/min for 5 min, then uniform velocity maintained at 0.1 mL/min. The process of blood withdrawal lasted more than 20 min and the blood was stored for serum tests. After being hypotensive for 40 min, the rats of both the ligation group and non-ligation group were resuscitated by Ringer's solution for as much as 3 times the lost blood volume for more than 30 min, then ligated through the right common carotid arteries and left jugular veins, and the incisions were then sutured. All the rats of the three groups received an abdominal operation with a vertical incision of 4 cm in length. The end of the mesentery was exposed. The mesenteric lymphatic ducts, which ran along the superior mesenteric artery, were separated. The rats of the ligation groups were additionally ligated at the mesenteric lymph duct, whereas sham and non-ligation groups were not, these were only threaded with cotton below the mesenteric lymph duct and then the abdomen was closed. After 6 h, LPS (O111:B4, Sigma) was injected intraperitoneally at a dose of 4 mg/kg, and 5% glucose saline at 20 mL/kg every 2 h until after 22 h for supportive therapy. After 24 h, all rats were anesthetized again with sodium pentobarbital (25 mg/kg), and their left common carotid arteries were cannulated to sample blood for serum tests by centrifugation. Then all rats were sacrificed and the organs were taken out, including the kidney, liver, lung and heart. Microscopic sections were made. The sera and tissue homogenate were stored at -26°C for later use.

Biochemical indexes and pathomorphology

The biochemical indexes of hepatic and renal function and myocardiac enzymes including the aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine (Cr), lactic acid dehydrogenase-1 (LDH-1) were determined before and after experimentation by an automatic biochemical analyzer type of Aeroset. The kidney, liver, lung and heart tissues were fixed by formaldehyde, embedded in paraffin, sliced and stained by HE. The pathological changes of the tissues were observed.

Determination of $\text{NO}_2^-/\text{NO}_3^-$, NOS, SOD and MDA in serum and tissue homogenate

The concentrations of $\text{NO}_2^-/\text{NO}_3^-$, nitric oxide synthase (NOS), inducible nitric oxide synthase (iNOS), superoxide dismutase (SOD) and malondialdehyde (MDA) in serum and tissue homogenate were determined by nitrate reductase method, chemical chromogenic reaction, modified TBA microdetermination, xanthinoxidase method (Naijing Jiancheng, China).

Statistical analysis

All data were expressed as mean \pm SD. SPSS software (Version 11.0 for Windows 98) was employed, one-way analysis of variance was used between groups, paired *t* test within group, and χ^2 test for survival analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Survival rate of two-hit rats

In the process of the two-hit for 24 h, the survival rates of rats in the sham group, non-ligation group and ligation group were 100% (15/15), 53.3% (8/15), 73.3% (11/15) respectively, with a significant difference between the three groups ($\chi^2 = 8.904$, $P < 0.05$).

Effect of mesenteric lymph duct ligation on biochemical indexes in two-hit rats

There was no obvious difference between the three groups in the serum biochemical indexes of liver, kidney and cardiac muscle function before experimentation, as well as before and after experimentation in the sham group. The levels of AST, ALT, BUN, Cr and LDH-1 in the ligation group and non-ligation group were obviously higher than that in the sham group after experimentation, as well as before experimentation ($P < 0.01$, $P < 0.05$). The levels of ALT, BUN and Cr in the ligation group were obviously lower than that in non-ligation group ($P < 0.01$, Table 1).

Pathomorphologic changes of the vital organs

Kidney: The structure of the glomerulus and renal tubule, proximal and distal convoluted tubes were seen clearly in the sham group (Figure 1A). Fibrinoid necrosis of capillary vessels of the glomerulus, plasma protein precipitation in the glomerular capsule, and necrosis in some renal tubules could be observed in the non-ligation group (Figure 1B). There was fibrinoid necrosis on the capillary wall of the glomerulus in the ligation group, but only a few protein casts in the renal tubule (Figure 1C).

Lung: In the sham group, the structure of the alveolus was normal. The alveolar wall was thin. There were alveolar epithelial cells on the surface. Capillary vessels of the alveolar wall were not dilated or congested. There was no exudate in the alveolar cavity (Figure 2A). In the non-ligation group, severe monopyrenous cell hyperplasia and hemorrhage in the alveolar gap could be seen, leading to atelectasis and some alveolar emphysematous change (Figure 2B). In the ligation group, there were hyperplasia of monopyrenous cells and hemorrhage in the alveolar

Table 1 Effect of mesenteric lymph duct ligation on biochemical indexes of two-hit rats (mean \pm SD)

Group	<i>n</i>		AST (nkat/L)		ALT (nkat/L)		BUN (mmol/L)		Cr (μ mol/L)		LDH-1 (nkat/L)	
	Pre-exp	Post-exp	Pre-exp	Post-exp	Pre-exp	Post-exp	Pre-exp	Post-exp	Pre-exp	Post-exp	Pre-exp	Post-exp
Sham	15	15	1767 \pm 333	1917 \pm 550	533 \pm 150	583 \pm 283	7.4 \pm 1.0	8.5 \pm 1.6	47.7 \pm 22.1	50.9 \pm 31.7	4334 \pm 2033	4051 \pm 2567
Non-ligation	15	8	1817 \pm 633	5801 \pm 1900 ^{b,d}	550 \pm 133	4117 \pm 1450 ^{b,d}	7.5 \pm 0.9	21.8 \pm 3.5 ^{b,d}	47.5 \pm 18.9	213.9 \pm 81.2 ^{b,d,f}	4017 \pm 2017	7985 \pm 2267 ^{b,d}
Ligation	15	11	1667 \pm 483	5184 \pm 2300 ^{b,d}	500 \pm 167	2967 \pm 1217 ^{b,d,f}	7.8 \pm 1.1	15.5 \pm 5.6 ^{b,d,f}	43.3 \pm 18.4	142.0 \pm 66.1 ^{a,b,d}	4917 \pm 1767	7151 \pm 2250 ^{b,d}

^b*P* < 0.01 vs pre-exp; ^d*P* < 0.01 vs sham; ^a*P* < 0.05, ^f*P* < 0.01 vs non-ligation.

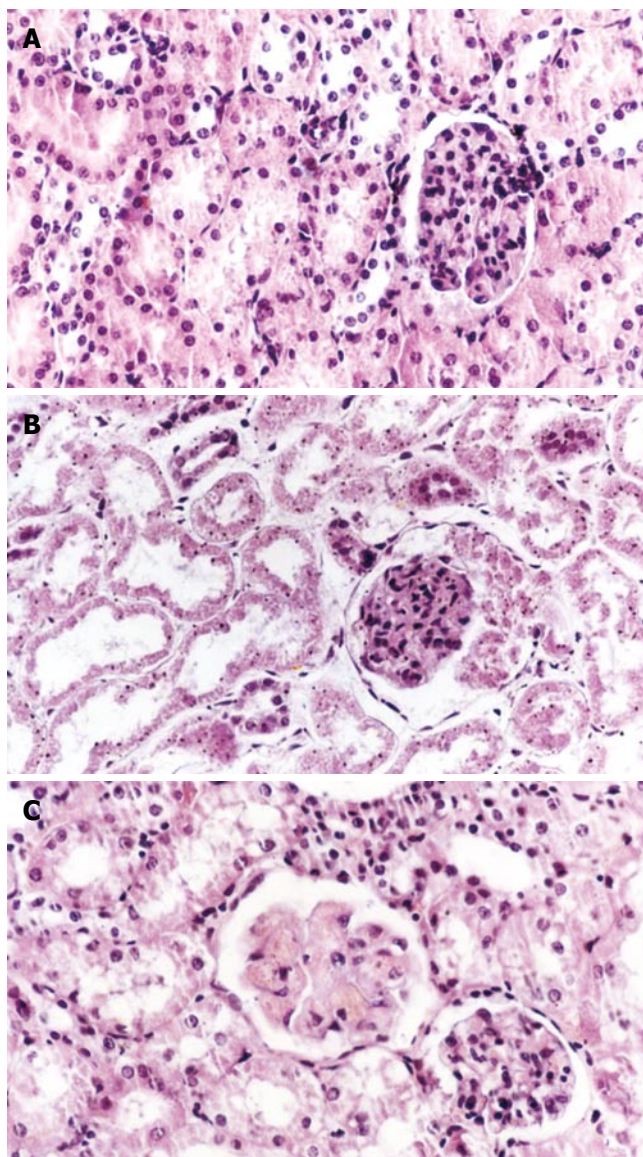


Figure 1 Effect of mesenteric lymph duct ligation on pathomorphology of kidney in MODS rats (HE \times 200). A: Sham; B: Non-ligation; C: Ligation.

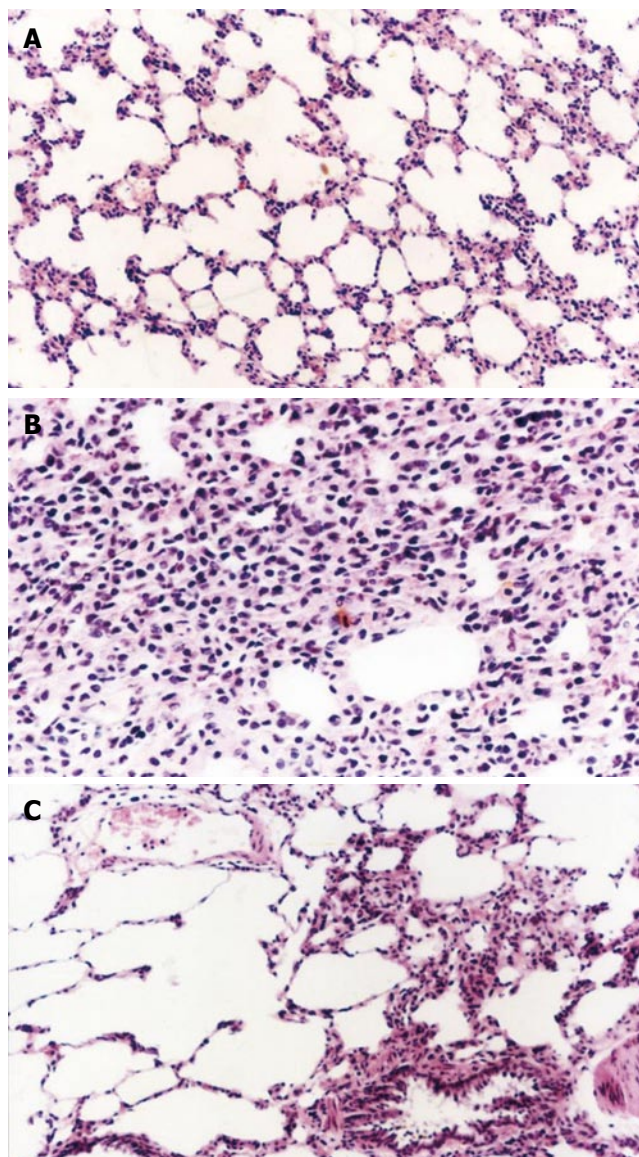


Figure 2 Effect of mesenteric lymph duct ligation on pathomorphology of lung in MODS rats (HE \times 100). A: Sham; B: Non-ligation; C: Ligation.

gap, resulting in widening of the alveolar gap and atrophy of alveoli. The surrounding alveoli were compensatorily emphysematous and the alveolar walls were thinner and broken. The local blood vessels were dilated and hematose (Figure 2C).

Liver: In the sham group, the central vein of the liver lobule was dilated slightly. The liver cells were arranged slightly disordered, but all were of the same size.

Their nucleoli were round and centrally located. Their karyotheca was clear (Figure 3A). In the non-ligation group, the liver cells were necrotic, with karyopycnosis, karyorrhexis, karyolysis and focal hemorrhage (Figure 3B). In the ligation group, the central vein of the liver lobule and the surrounding liver sinuses were obviously dilated and hematose. Liver cells arranged disorderly, but their conformations were normal (Nucleoli were of the same

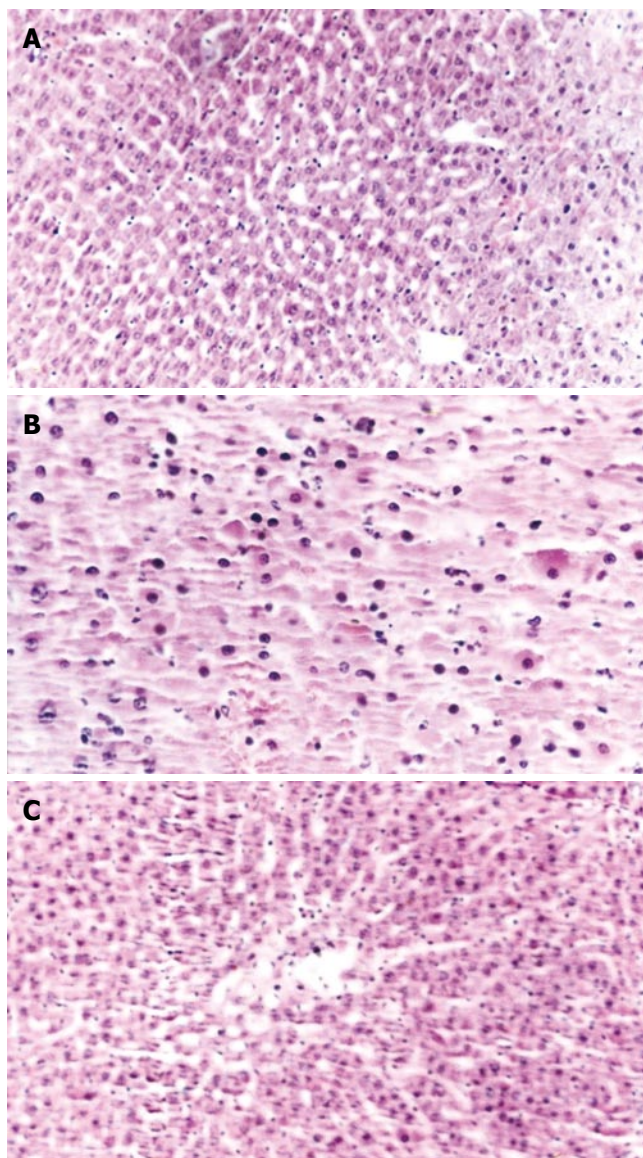


Figure 3 Effect of mesenteric lymph duct ligation on pathomorphology of liver in MODS rats (HE $\times 100$). A: Sham; B: Non-ligation; C: Ligation.

size, round and centered. The karyotheca was clear). Only some liver cells were necrotic, such as karyopycnosis, karyorrhexis and karyolysis (Figure 3C).

Cardiac muscle: In the sham group, the structure of the cardiac muscle fiber was normal. Cell nucleolus was centered, karyotheca was clear, and cardiac muscle cells were of the same size (Figure 4A). In the non-ligation group, there were coagulation necroses in the cardiac muscle fiber. Only the nucleolus of the myocardial mesenchyme was left, with focal sarcoplasm lysis and necrosis. A few structures of mesenchyme were seen (Figure 4B). In the ligation group, there were focal sarcoplasm coagulations in the cardiac muscle fiber. The nucleolus disappeared and the myocardial mesenchyme had focal infiltration of inflammatory cells (Figure 4C).

Effects of mesenteric lymph duct ligation on NO content and NOS activity in two-hit rats

Effects on NO content and NOS activity in serum: The contents of $\text{NO}_2^-/\text{NO}_3^-$, NOS and iNOS in serum

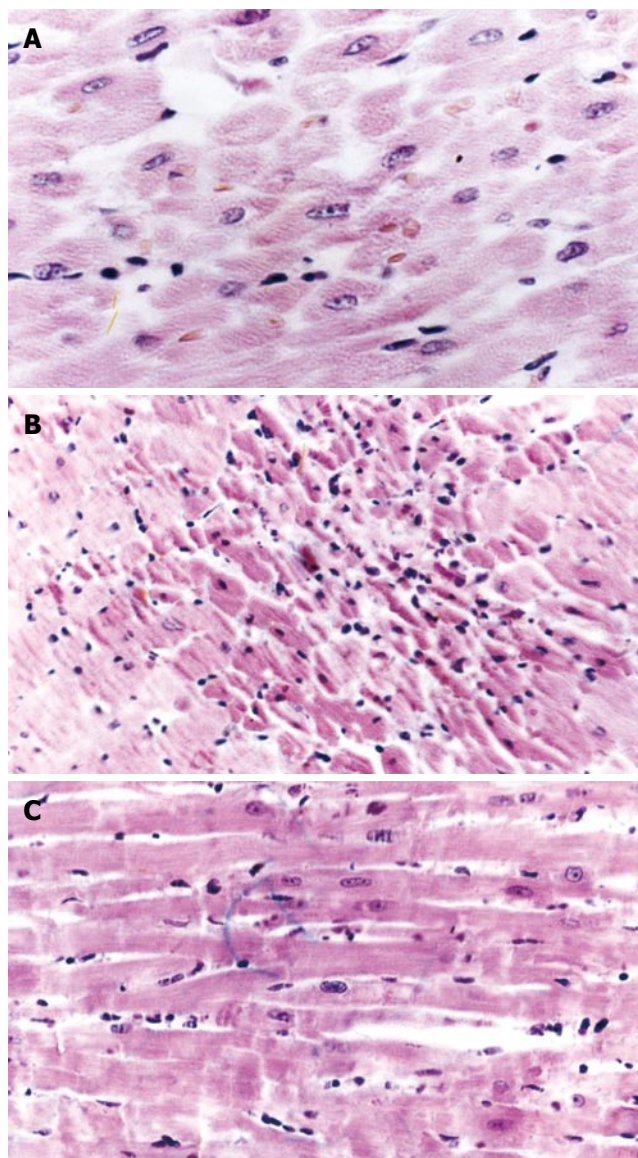


Figure 4 Effect of mesenteric lymph duct ligation on pathomorphology of heart in MODS rats (HE $\times 200$). A: Sham; B: Non-ligation; C: Ligation.

had no significant difference between the three groups before experimentation and in the sham group between pre- and post-experimentation. After two-hit, the contents of $\text{NO}_2^-/\text{NO}_3^-$, NOS and iNOS in the serum of the non-ligation group were significantly higher than that before experimentation and in the sham group ($P < 0.01$). The contents of $\text{NO}_2^-/\text{NO}_3^-$ and NOS of the ligation group were significantly higher than that in the sham group ($P < 0.05$), but were significantly lower than in the non-ligation group ($P < 0.01$, Table 2).

Effects on NO content and NOS activity in tissue homogenate: Compared with the sham group, the contents of $\text{NO}_2^-/\text{NO}_3^-$ in intestine, kidney, liver, lung and heart homogenates in the non-ligation group were significantly increased ($P < 0.01$), as well as in kidney, lung and heart homogenate in the ligation group, but no significant difference existed in intestine and liver ($P > 0.05$). The contents of $\text{NO}_2^-/\text{NO}_3^-$ in intestine, kidney and liver homogenate of the ligation group were significantly lower than that of the non-ligation group ($P < 0.01$), but

Table 2 Effect of mesenteric lymph duct ligation on NO₂⁻/NO₃⁻, NOS and iNOS in serum of two-hit rats (mean ± SD)

Group	<i>n</i>		NO ₂ ⁻ /NO ₃ ⁻ (mmol/L)		NOS (nkat/L)		iNOS (nkat/L)	
	Pre-exp	Post-exp	Pre-exp	Post-exp	Pre-exp	Post-exp	Pre-exp	Post-exp
Sham	15	15	84.2 ± 48.4	79.4 ± 33.3	245382 ± 132026	219544 ± 78349	87851 ± 43842	71681 ± 37647
Non-ligation	15	8	88.6 ± 38.5	217.1 ± 48.1 ^{b,d}	251550 ± 69347	389411 ± 90851 ^{b,d}	91685 ± 22338	175035 ± 45009 ^{b,d}
Ligation	15	11	86.3 ± 41.7	159.9 ± 44.1 ^{b,d,f}	265053 ± 102520	312562 ± 129193 ^a	85017 ± 42842	109355 ± 24005 ^f

^b*P* < 0.01 *vs* pre-exp; ^a*P* < 0.05, ^d*P* < 0.01 *vs* sham; ^f*P* < 0.01 *vs* non-ligation.

Table 3 Effect of mesenteric lymph duct ligation on NO₂⁻/NO₃⁻, NOS and iNOS in homogenate of two-hit rats (mean ± SD)

Group (<i>n</i>)		Intestine	Kidney	Liver	Lung	Heart
NO ₂ ⁻ /NO ₃ ⁻ (μmol/g)	Sham (15)	3.3 ± 1.0	1.4 ± 0.3	1.6 ± 1.0	1.4 ± 0.5	2.0 ± 0.4
	Non-ligation (8)	9.1 ± 2.9 ^b	5.1 ± 1.4 ^b	4.0 ± 1.2 ^b	3.2 ± 0.9 ^b	3.5 ± 0.9 ^b
	Ligation (11)	4.3 ± 2.2 ^d	2.9 ± 0.7 ^{b,d}	2.2 ± 0.8 ^d	2.7 ± 0.7 ^a	3.1 ± 0.6 ^a
NOS (nkat/g)	Sham (15)	20.7 ± 4.7	11.2 ± 4.5	12.2 ± 4.3	0.62 ± 0.17	24.8 ± 3.7
	Non-ligation (8)	30.2 ± 9.7 ^a	15.5 ± 6.3 ^b	18.8 ± 8.2	0.84 ± 0.32	28.5 ± 5.1
	Ligation (11)	27.0 ± 6.8	13.7 ± 4.5	13.5 ± 3.8	0.73 ± 0.15	26.5 ± 5.1
iNOS (nkat/g)	Sham (15)	9.8 ± 2.2	6.2 ± 2.8	5.8 ± 2.8	4.0 ± 1.0	11.0 ± 4.0
	Non-ligation (8)	15.3 ± 3.8 ^b	7.7 ± 3.3	8.0 ± 5.0	7.0 ± 3.2	15.2 ± 6.0
	Ligation (11)	12.0 ± 2.3	7.0 ± 4.0	7.0 ± 3.5	5.7 ± 1.3	12.0 ± 4.0

^a*P* < 0.05, ^b*P* < 0.01 *vs* sham; ^d*P* < 0.01 *vs* non-ligation.

Table 4 Effect of mesenteric lymph duct ligation on SOD and MDA in serum of two-hit rats (mean ± SD)

Group	<i>n</i>		SOD (nkat/L)		MDA (μmol/L)	
	Pre-exp	Post-exp	Pre-exp	Post-exp	Pre-exp	Post-exp
Sham	15	15	1732013 ± 134027	1687004 ± 100020	6.01 ± 1.53	6.82 ± 1.17
Non-ligation	15	8	1711342 ± 139195	1394446 ± 210542 ^{b,d}	5.55 ± 1.18	10.06 ± 1.28 ^{b,d}
Ligation	15	11	1664666 ± 116690	1610489 ± 149197 ^f	6.46 ± 1.48	8.52 ± 1.16 ^{b,d,f}

^b*P* < 0.01 *vs* Pre-exp; ^d*P* < 0.01 *vs* sham; ^f*P* < 0.01 *vs* non-ligation.

lung and heart homogenate had no significant difference between the two groups (*P* > 0.05). The NOS activity in intestine and kidney homogenate of the non-ligation group was significantly increased compared with the sham group (*P* < 0.01, *P* < 0.05), and that in liver, lung and heart homogenate seemed to be increased, but with no significant difference (*P* > 0.05). The iNOS activity in intestine homogenate of the non-ligation group was higher than that of the sham group (*P* < 0.01), but kidney, liver, lung and heart homogenate had no significant difference (*P* > 0.05). The NOS and iNOS activity in intestine, kidney, liver, lung and heart homogenate of the ligation group had no significant difference compared with the sham group and the non-ligation group (*P* > 0.05, Table 3).

Effects of mesenteric lymph duct ligation on SOD activity and MDA content in two-hit rats

Effects on SOD activity and MDA content in serum: The SOD activity and MDA content in serum had no significant difference in the three groups before

experimentation and in the sham group pre- and post-experimentation (*P* > 0.05). After experimentation, the MDA content of serum in the non-ligation group and ligation group were significantly increased than that of pre-experimentation and in the sham group (*P* < 0.01), but the MDA content in the ligation group was significantly lower than that of the non-ligation group (*P* < 0.01). The SOD activity in the non-ligation group was significantly lower than that of pre-experimentation, sham group and ligation group (*P* < 0.01), but SOD activity in the ligation group had no significant difference from pre-experimentation and in the sham group (*P* > 0.05, Table 4).

Effects on SOD activity and MDA content in tissue homogenate: The SOD activity of intestine homogenate in non-ligation group was significantly lower than that in sham group (*P* < 0.05), but SOD activity of intestine and heart in ligation group was higher than that in non-ligation group (*P* < 0.05), whereas in other tissue homogenates it had no significant difference (*P* > 0.05). The MDA content of intestine, kidney and liver homogenate in the non-

Table 5 Effect of mesenteric lymph duct ligation on SOD and MDA in homogenate of two-hit rats (mean \pm SD)

Group (n)		Intestine	Kidney	Liver	Lung	Heart
SOD (nkat/g)	Sham (15)	442255 \pm 77349	273221 \pm 43009	205374 \pm 35840	226212 \pm 38174	300227 \pm 58011
	Non-ligation (8)	330399 \pm 77516 ^a	268720 \pm 20504	204874 \pm 20838	175035 \pm 44676	241882 \pm 56178
	Ligation (11)	467594 \pm 74182 ^c	256885 \pm 28172	212376 \pm 35674	192205 \pm 47009	339235 \pm 49510 ^c
MDA (μ mol/g)	Sham (15)	2.26 \pm 0.65	1.62 \pm 0.45	1.66 \pm 0.52	1.12 \pm 0.25	1.33 \pm 0.36
	Non-ligation (8)	3.28 \pm 0.58 ^a	2.28 \pm 0.16 ^a	2.27 \pm 0.28 ^a	1.34 \pm 0.33	1.84 \pm 0.62
	Ligation (11)	2.81 \pm 0.59	2.22 \pm 0.58 ^a	2.24 \pm 0.34 ^a	1.32 \pm 0.20	1.52 \pm 0.40

^a $P < 0.05$ vs sham; ^c $P < 0.05$ vs non-ligation.

ligation group and of kidney and liver homogenate in the ligation group were higher than that in the sham group ($P < 0.05$). The MDA content of lung and heart homogenate in non-ligation and of intestine, lung, heart homogenate in the ligation group had no significant difference from those of the sham group ($P > 0.05$), and no significant difference existed between the non-ligation group and the ligation group, either ($P > 0.05$, Table 5).

DISCUSSION

The occurrence of MODS is related to the pathogenic causes such as trauma, shock, ischemia-reperfusion injury and endotoxemia, *etc*^[14]. The various inflammatory cells are continuously stimulated and activated by pathogenic factors, producing and releasing mass cytokines and inflammatory mediators. There are complex chain reactions and cytokine networks between cytokines, cytokine and inflammatory mediators^[12,15]. In the process of production and release, they stimulate each other, act synergistically, or inhibit and counteract with each other, producing a cytokine cascade effect, and gradually an amplified effect on every organ and system, leading to the non-specific injury of tissues, damage of organs, and finally the occurrence of systemic inflammatory response syndrome (SIRS)^[1,16-19]. This is also the important pathophysiologic mechanism causing MODS. The MODS model can be made by one-hit and two-hit methods^[13]. In our study, the MODS rat model was successfully established by a two-hit method of hemorrhage/resuscitation and LPS, and the two-hit model showed different degrees of damage and dysfunction of liver, kidney and myocardium from pathomorphology and biochemical indexes of hepatic, renal function and myocardial enzyme at pre- and post-experimentation. Through this model, the effect of blocking intestinal lymphatic circulation in two-hit rats was studied.

In pathogenesis of diphasic type MODS, damage of the intestinal barrier function would induce intestinal endotoxemia and bacterial translocation from intestine^[14,21]. The circumfluence of intestinal and celiac contents has both blood and lymph routes^[22]. We investigated the importance of intestinal mesenteric lymph circumfluence in MODS pathogenesis in two-hit rats by ligating mesenteric lymphatic duct. The biochemical indexes of liver, kidney, and heart function before and after two-hit in three groups showed that after two-hit, the contents of AST, ALT, BUN, Cr and LDH-1 in the serum of the non-

ligation group and ligation group were obviously higher than that before experimentation and in the sham group. The contents of ALT, BUN and Cr in the ligation group were obviously lower than in the non-ligation group. All these suggest that the ligation of mesenteric lymph duct had a protective effect on the function of liver, kidney and heart because the ligation led to a decrease of the entry of intestinal bacteria and endotoxins into blood. A pathomorphologic study showed that the cellular structures of the kidney, lung, liver and heart tissues in the sham group were normal, while congestion, degeneration and necrosis were found in organs of the non-ligation group, and only mild lesions could be found in the ligation group. It suggests that the ligation of mesenteric lymph duct had a protective effect on the liver, kidney, lung, and heart in two-hit rats. In addition, ligating mesenteric lymph duct could help resuscitation from two hits of hemorrhage and LPS, improve the survival rate; meanwhile it also confirmed the protective effects of mesenteric lymph diversion.

After LPS was injected into the abdominal cavity of the wounded rats, the histology of lymphatic tissue showed that there existed a lymphatic micronet at the visceral and parietal peritoneum; the largest surface area was those covering the digestive tube. These lymphatic capillary nets formed the lymphatic plexus, which sent out collecting lymphatic vessels to regional nodes. Mesenteric lymph duct is a chief excurrent passage. When the function of intestinal barrier was damaged, the toxic substance also could be translocated through mesenteric lymph duct^[23]. By ligation of mesenteric lymph duct, the blockage of the lymph stream from intestines and peritoneum would make LPS (MW = 30 kD) and other intestinal poisonous substances difficult to get into mesenteric lymph duct, thus relieving the morphologic damage and functional disorder of many organs.

Intestinal barrier dysfunction and intestinal inflammation caused by bacteria/endotoxin translocation (BET) are the main reason for pyotoxemia and MODS in severe inflammatory patients with unclear origin^[24-27]. In recent years^[28,29], some scholars also put forward that the intestines are the "center" organ for MODS and the chief cell origin for TNF α . Thus the intestines are not only the target organs of damage, but also the main links in MODS. In our experiment, probably *via* blocking the linkage function of the intestines as center organs, ligation of mesenteric lymph duct exerted the protective effect on several organs. From the contents of NO, NOS, iNOS

and MDA in serum and tissue homogenate, it indicates that the cytokine cascade reaction was induced by two-hits of hemorrhage and LPS. It also suggests that ligation could reduce iNOS, NOS, NO and free radicals produced and released by the intestines into the systemic circulation through lymph ducts, thus alleviating their damage to the organs.

LPS is a main component of the outer cell wall structure of Gram-negative bacteria. Although micro-lymph ducts have the function of absorbing macromolecular substances actively, in this experiment, LPS was injected into the abdominal cavity, but not the digestive cavity directly. Therefore, the majority of LPS not only could be absorbed *via* the visceral peritoneum of the digestive tube which has the largest area, but also could be absorbed *via* micro-lymph ducts of other visceral peritoneum, then through lymphatic plexus which send out collecting lymphatic vessels to regional nodes, or through microvessels into blood. This might be the reason for high MDA content in liver and kidney homogenate of the ligation group, which was significantly higher than that of the sham group, but not significantly different from the non-ligation group.

In conclusion, ligation of mesenteric lymph duct has a protective effect in two-hit rats. It could reduce the production of iNOS, synthesis of NO, release of free radicals, and consumption of SOD, thus improving the disturbance of organic function and pathomorphological changes, and relieving the damage of two-hits on kidney, liver, lung, heart and intestine. It indicates that mesenteric lymph plays an important role in cytokine production and transportation of inflammatory response mediators in two-hit rats. Blockade of mesenteric lymph might be a new approach in prevention and treatment of two-hits.

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Effect of nutritional counselling on hepatic, muscle and adipose tissue fat content and distribution in non-alcoholic fatty liver disease

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Abstract

AIM: To assess the effectiveness of the current UK clinical practice in reducing hepatic fat (IHCL).

METHODS: Whole body MRI and ^1H MRS were obtained, before and after 6 mo nutritional counselling, from liver, soleus and tibialis muscles in 10 subjects with non-alcoholic fatty liver disease (NAFLD).

RESULTS: A 500 Kcal-restricted diet resulted in an average weight loss of 4% (-3.4 kg,) accompanied by significant reductions in most adipose tissue (AT) depots, including subcutaneous (-9.9%), abdominal subcutaneous (-10.2%) and intra-abdominal-AT (-11.4%). Intramyocellular lipids (IMCL) were significantly reduced in the tibialis muscle (-28.2%). Decreases in both IHCL (-39.9%) and soleus IMCL (-12.2%) content were also observed, although these were not significant. Several individuals showed dramatic decreases in IHCL, while others paradoxically showed increases in IHCL content. Changes in body composition were accompanied by improvements in certain liver function tests: serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Significant correlations were found between decreases in IHCL and reductions in both intra-abdominal and abdominal

subcutaneous AT. Improvements in liver function tests were associated with reductions in intra-abdominal AT, but not with changes in IHCL.

CONCLUSION: This study shows that even a very modest reduction in body weight achieved through lifestyle modification can result in changes in body fat depots and improvements in LFTs.

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Key words: Intra-abdominal adipose tissue; Intrahepatic fat; Intramyocellular lipids; Weight loss; Magnetic resonance imaging

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INTRODUCTION

The incidence of non-alcoholic fatty liver disease (NAFLD) has increased rapidly over the last few years and is now one of the commonest causes of abnormal liver function test (LFT) results in patients presenting to hepatology clinics in both Europe and the USA^[1]. Hepatic steatosis may be prevalent in more than 30% of the population^[2,3]. NAFLD encompasses a wide spectrum of liver diseases, from mild fatty infiltration, through to steatohepatitis, cirrhosis and fibrosis^[4]. One of the major factors thought to be responsible for fat accumulation in the liver is obesity^[3], with NAFLD being a key feature of insulin resistance and the metabolic syndrome^[5]. Treatment is currently limited, although lifestyle modification including dietary change and increased exercise to promote weight loss are thought beneficial^[6].

The nature and rapidity of weight loss has been shown to be important^[7]. Previous studies have used gastric banding^[8,9], or very low calorie diets^[7,10] to promote significant, or rapid weight loss, resulting in reduced

intrahepatocellular lipid (IHCL) content. However, rapid reduction in weight may worsen underlying inflammation and fibrosis in patients with non-alcoholic steatohepatitis (NASH)^[7,9]. Several studies have shown that liver fat content in NAFLD can be reduced through more moderate weight loss, achieved by dietary restriction, either alone^[11,12], in combination with exercise^[13-17], or using pharmacological intervention^[18-26]. To date, no study has looked at the effects of first line treatment in clinical management algorithms offered to most people attending outpatient clinics with NAFLD. The aim of this study was therefore to assess the impact of current United Kingdom nutritional clinical practice to reduce body adiposity and its impact on liver fat content and measures of hepatic function.

MATERIALS AND METHODS

Written informed consent was obtained from all volunteers. Permission for this study was obtained from the Ethics Committee of Hammersmith Hospital, Imperial College London, (Rec. 93/4047; 93/3995). The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Subjects

Ten patients, referred to the Hepatology Outpatient Clinics at the Hammersmith and St Mary's Hospitals in London, were recruited (ST-R). All had unexplained abnormal liver function tests (LFTs) as a reason for referral with raised serum aspartate transaminase (AST) and/or gamma-glutamyl transpeptidase levels (γ GT). All were clinically obese with a mean body mass index of $31.6 \pm 4.6 \text{ kg/m}^2$. Three subjects had type II diabetes diagnosed within the last 3 years, two were treated with diet only, and one subject took metformin. Five had a history of dyslipidaemia. No patient drank alcohol in excess of 20 g/d and none had a history of excess alcohol consumption. No co-existing reason for the LFT abnormalities was found on screening for viral hepatitis or autoimmune liver disease. Serum copper, caeruloplasmin and iron studies were normal in each patient. All had increased echogenicity on hepatic ultrasound examination, compatible with hepatic steatosis. This was confirmed on subsequent liver biopsy in four of the subjects (RDG). The characteristics of the study group at baseline are shown in Table 1.

Dietary Intervention

All patients were referred to the Hammersmith Hospital 'Lifestyle Clinic'. The aim of the treatment was a gradual weight loss of 5%-10% of initial body weight within 6 mo. Subjects attended seven appointments with a registered dietician (AEB) over 6 mo, with fortnightly phone calls between appointments. Reported energy intakes using 3 d diaries were $2464 \pm 66 \text{ kcal}$, (of which $46\% \pm 4\%$ carbohydrate, $35\% \pm 3\%$ fat, $18\% \pm 1\%$ protein), in line with typical British diets. All subjects were sedentary at baseline scoring 7.1 ± 0.4 using Baecke activity questionnaires. Subjects were given advice on modifying their diets, which centred on behaviour change around

Table 1 Anthropometry results before and after dietary intervention mean \pm SD

	Pre-diet (<i>n</i> = 10)	Post-diet (<i>n</i> = 10)	<i>P</i>
Weight (kg)	96.3 (87.9-105.6)	93.0 (83.9-103.0)	0.006
BMI (kg/m ²)	31.3 (28.3-34.9)	30.2 (27.2-33.9)	0.006
Waist circumference (cm)	114.6 (107.8-121.4)	107.9 (101.6-114.3)	0.001
Systolic BP (mmHg)	133.4 (117.0-149.8)	129.8 (121.3-138.2)	0.62
Diastolic BP (mmHg)	79.4 (76.5-82.3)	77.6 (71.9-83.8)	0.50
Pulse (bpm)	67.3 (60.6-75.2)	66.3 (55.3-79.9)	0.60

Significance taken as $P < 0.05$. BMI: Body mass index; BP: Blood pressure; bpm: Beats per minute.

dietary intake in accordance with Hammersmith Hospitals Dietetic Department policy^[27]. The aim was to induce a 500 kcal energy deficit in the diet. Activity was encouraged in the form of walking using the "10 000 steps a day" campaign and pedometers were advised to aid compliance.

Biochemistry

Fasting blood samples were obtained for measurement of glucose, insulin, cholesterol, triglycerides and glycosylated haemoglobin (HbA_{1c}). AST, ALT, and γ GT were determined as recommended by the European Committee for clinical laboratory standards.

Anthropometry

Body weight was measured to the nearest 100 g. Height was measured using a stadiometer to the nearest centimetre. Waist circumference was measured mid-way between the lowest rib and the iliac crest^[28].

Total body adipose tissue content

Rapid T₁-weighted MR images (TR 36 ms, TE 14 ms) were acquired as previously described^[29]. Subjects lay in a prone position with arms straight above the head, and were scanned from fingertips to toes, acquiring 10 mm-thick transverse images with 30 mm gaps between slices in the arms and legs, and 10 mm gaps between slices in the trunk. Images were analysed using SliceOmatic (Tomovision, Montreal, Quebec, Canada). Total and regional adipose tissue (AT) volumes were measured^[29].

MRS of the liver

¹H MR spectra were acquired on a 1.5T Eclipse multi-nuclear system (Phillips Medical Systems, Cleveland, Ohio) using a flexible body coil. Spectra were obtained from the right lobe of the liver using a PRESS sequence (TR 1500 ms, TE 135 ms) without water saturation and with 128 signal averages. Intrahepatocellular lipids (IHCL) were measured relative to liver water content as previously described^[30].

MRS of muscle

Intramyo cellular lipids (IMCL) were measured in the soleus (S-IMCL) and tibialis (T-IMCL) muscles by ¹H MRS (TR 1500 ms, TE 135 ms, 256 averages). IMCL

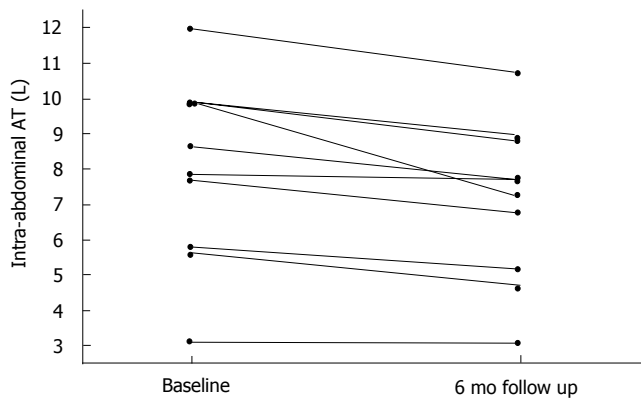


Figure 1 Changes in intra-abdominal adipose tissue content following six months dietary intervention.

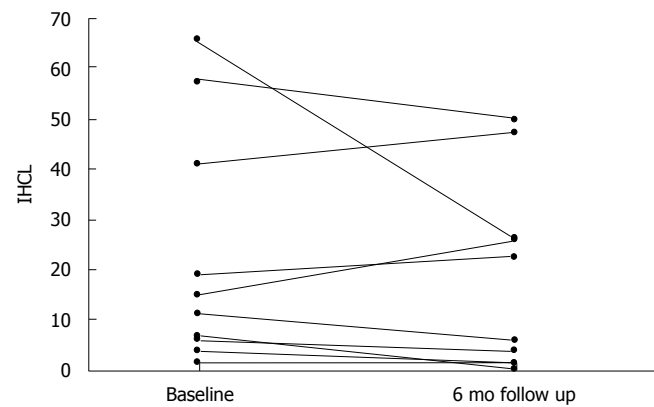


Figure 2 Changes in IHCL content following six months dietary intervention.

Table 2 Whole body MRI before and after dietary manipulation ($n = 10$, geometric mean)

Adipose tissue (L)	Pre-diet	Post-diet	Change (%)	<i>P</i> (<i>t</i> -test)
Total	38.8 (32.1 to 46.9)	35.1 (28.5 to 43.4)	-9.5 (-14.4 to -4.4)	0.003
Subcutaneous	25.3 (20.8 to 30.8)	22.8 (18.1 to 28.7)	-9.9 (-15.5 to -3.9)	0.006
Internal	13.3 (10.6 to 16.7)	12.1 (9.6 to 15.2)	-9.3 (-14.1 to -4.2)	0.003
Sc abdominal	7.6 (5.8 to 10.1)	6.9 (5.1 to 9.3)	-10.2 (-18.6 to -0.9)	0.04
Sc peripheral	17.6 (14.9 to 20.8)	15.9 (12.9 to 19.5)	-9.7 (-14.9 to -4.3)	0.003
Intra-abdominal	7.6 (5.7 to 10.0)	6.7 (5.1 to 8.7)	-11.4 (-16.5 to -6.0)	0.001
Non-abdominal internal	5.7 (4.7 to 6.9)	5.4 (4.4 to 6.6)	-5.4 (-14.4 to 4.5)	0.24

t-tests were performed on log-transformed data, significance taken as $P < 0.05$. AT = adipose tissue; Sc = subcutaneous.

were measured relative to total muscle creatine signal, as previously described^[31].

Statistical analysis

Statistical advice was provided by Dr Caroline Doré, MRC Clinical Trials Unit, London UK. The distribution of the data was tested for using the Shapiro-Wilk normality test. Normally distributed data are expressed as mean and 95% confidence interval (CI). Log₁₀ transformation was used to correct variables that were not normally distributed. Results for log-transformed variables are presented as geometric mean and 95% CI. Comparison before and after lifestyle intervention were tested using a paired *t*-test. Associations between variables were assessed using Pearson's correlation coefficients. The level of significance was set at 5%. Stepwise multiple regression analysis was performed to predict changes in AST, ALT and IHCL. Given the small number of patients, only forward variable selection was used. Data were analysed using Unistat version 5.5 (Unistat Ltd, London, UK) and Stata (StataCorp 2001 Stata Statistical Software, Release 7.0; Stata Corporation, College Station, Texas, USA).

RESULTS

All patients who participated in this study lost weight, with a mean loss of -3.5 kg (range -0.6 to -10.0 kg, $P = 0.006$). Total AT content was also reduced, with a mean reduction of -3.5 litres (range -0.75 to -10.43 litres, $P = 0.003$). There was also a significant reduction in waist circumference -6.6

cm (range -2.5 to -13.0 cm, $P = 0.001$) (Table 1). There were significant decreases in most AT depots following this life style intervention (Table 2). The largest decrease was found in intra-abdominal AT (-11.4%) (Figure 1), with slightly smaller quantities of subcutaneous AT lost from abdominal (-10.2%) and peripheral areas (-9.7%). There was a strong relationship between the amount of abdominal AT lost subcutaneously and internally ($r = 0.81$, $P < 0.01$).

A reduction in AST and ALT was observed, although the latter did not reach significance (Table 3). There was no significant change in γ GT. HbA_{1c} was also significantly reduced following the lifestyle intervention, suggesting an improvement in glycemic control. This may also be inferred by an improvement in insulin sensitivity observed in some individuals (HOMA %S), although as a group the increase did not reach significance.

The ¹H MRS findings are shown in Table 4. Although, seven of the 10 subjects showed marked reductions in IHCL (-57.2%), three subjects showed increases (33.2%), despite no significant difference between the groups in terms of weight loss (Figure 2). Thus, as a group the decrease in IHCL did not reach significance (-39.9%, $P = 0.12$). Interestingly, there was a significant correlation between changes in IHCL, weight loss ($r = 0.74$, $P < 0.01$) and intra-abdominal AT changes ($r = 0.83$, $P < 0.01$). This relationship was also significant for changes in IHCL and abdominal subcutaneous depot ($r = 0.76$, $P < 0.01$). There was a significant decrease in T-IMCL levels, but not for S-IMCL. Changes in T-IMCL were related to decreased

Table 3 Serum biochemistry results before and after dietary manipulation ($n = 10$)

Biochemistry	Pre-diet	Post-diet	P
Glucose (mmol/L)	5.7 (5.2-6.2)	5.5 (4.8-6.1)	0.55
Insulin (mIU/L)	13.4 (8.4-18.4)	13.9 (8.2-19.5)	0.82
HOMA %S	424.04 (225.2-737.5)	540.1 (38.0-1400.8)	0.33
HOMA %B	28.5 (17.5-43.4)	25.2 (16.2-37.3)	0.50
HbA _{1c}	6.0 (5.7-6.3)	5.6 (5.2-6.1)	0.03
Cholesterol (mmol/L)	5.3 (4.6-6.1)	5.0 (4.6-5.5)	0.24
Total Chol:HDLc	4.4 (3.7-5.4)	4.0 (3.5-4.7)	0.22
TG (mmol/L)	1.9 (1.3-2.6)	1.9 (1.4-2.4)	0.88
AST (U/L)	32.2 (26.5-37.9)	28.4 (24.0-32.8)	0.02
ALT (U/L)	42.4 (29.1-55.7)	36.6 (22.5-50.7)	0.08
γ GT (U/L)	39.0 (18.0-60.0)	40.2 (21.6-58.8)	0.34

HOMA: Homeostatic model assessment, %S estimates insulin sensitivity; %B estimates β -cell function; Chol: Cholesterol; HDL: High density lipoprotein; TG: Triglyceride; AST: Serum aspartate aminotransferase; ALT: Alanine aminotransferase; γ GT: Gamma-glutamyl transpeptidase.

intra-abdominal AT ($r = 0.73$, $P < 0.02$), whereas changes in S-IMCL were related to decreased subcutaneous AT in both abdominal ($r = 0.81$, $P < 0.01$) and peripheral regions ($r = 0.69$, $P < 0.05$).

Changes in LFTs showed significant association with alterations in body composition, (Table 5). A significant correlation was found between reduction in intra-abdominal AT and changes in both ALT ($r = 0.83$, $P < 0.01$) and AST ($r = 0.71$, $P < 0.05$). A weaker correlation was found between changes in ALT and IHCL ($r = 0.62$, $P = 0.05$). Stepwise multiple regression analyses were performed to predict changes in AST, ALT and IHCL. Variables considered for inclusion in this model were changes in abdominal and peripheral subcutaneous AT, intra-abdominal and non-abdominal internal AT, weight and IMCL in the soleus and tibialis muscles. Only changes in intra-abdominal AT were able to predict changes in AST, ALT and IHCL. Changes in AST and ALT were unable to predict changes in IHCL.

DISCUSSION

In this study we have shown that modest weight loss, obtained through routinely available hospital dietetic clinical care, can have worthwhile effects on whole body adiposity, hepatic and muscle fat content and LFTs in NAFLD patients. These findings indicate that current UK clinical practice is effective in promoting a lifestyle change that has a positive effect on reducing adiposity. Huang *et al* used a similar nutritional method to that employed in the current study to promote weight loss and looked at changes in liver histology on biopsy. They found histological improvement in patients with a weight loss of 7%, but not in those who lost only 2%^[12]. Similar findings were reported by Tikkainen *et al* in patients with an 8% weight loss^[11]. Tamura *et al* using diet with or without exercise in a strictly controlled study, reported decreases in body fat of 9.6% and 8.2%, with a 25%-30% reduction in IHCL. IMCL also decreased, but only with exercise^[13]. Hickman, reported an improvement in steatosis and liver

Table 4 Hepatic and Muscle fat before and after dietary modification ($n = 10$, geometric mean, t -test)

	Pre-diet	Post-diet	Change (%)	P
¹ IHCL	13.3 (5.8-30.9)	8.0 (2.4-26.4)	-39.9 (-69.5-18.4)	0.12
¹ IMCL soleus	19.6 (10.3-37.2)	17.2 (10.8-27.3)	-12.2 (-53.0-64.2)	0.65
¹ IMCL tibialis	11.6 (7.6-17.7)	8.3 (5.4-12.8)	-28.2 (-48.5-0.03)	0.05

¹Measured in arbitrary units. IHCL: Intrahepatocellular lipid content; IMCL: Intramyocellular lipid content.

Table 5 Relationship between changes in liver biochemistry and adiposity

Change	Hepatic fat		AST		ALT	
	r	P	r	P	r	P
Body weight	0.74	0.01	0.16	0.7	0.32	0.4
Total AT	0.70	0.02	0.42	0.22	0.49	0.15
Abdominal sc AT	0.76	0.01	0.57	0.09	0.54	0.11
Peripheral sc AT	0.45	0.2	0.15	0.7	0.17	0.6
Intra-abdominal AT	0.83	0.003	0.71	0.02	0.83	0.003
Non-abdominal internal AT	0.28	0.4	0.23	0.5	0.35	0.3
hepatic fat	-	-	0.51	0.13	0.62	0.05
Soleus IMCL	0.50	0.14	0.27	0.4	0.21	0.6
Tibialis IMCL	0.53	0.12	0.42	0.22	0.56	0.09

Pearson product movement correlation coefficients (r) for the relationship between AST, ALT and hepatic fat and measures of total and regional adiposity and IMCL, performed using log transformed data. AT: Adipose tissue; IMCL: intramyocellular lipid content; Sc: Subcutaneous.

histology following 12 wk of diet and exercise, with a weight loss of 6.6%^[14,15]. In our study, subjects lost on average 4% of their body weight, with variable effects on IHCL and IMCL levels. It is possible that a greater weight loss than this is necessary to have a significant effect on IHCL, or it may be that the nature/stage of the disease is important for overall reduction in steatosis. For example, in the present study, while several individuals had dramatic decreases in IHCL, others paradoxically showed increases in IHCL. The reasons for this are not immediately apparent. Recent studies have suggested that IHCL content may be altered by a single meal^[32], however we scanned our subjects following overnight fast, to minimise any potential effects. There were no obvious phenotypic or clinical differences between those subjects who responded to the weight loss with reductions in IHCL and the non-responders, who lost similar levels of body weight, but increased their IHCL content. However, where biopsy data were available (4/10 subjects), responders showed mild steatohepatitis, whereas non-responders additionally showed signs of more severe inflammation and fibrosis. Thus, IHCL reduction, as a result of dietary intervention, may be hampered in individuals whose fatty infiltration has already begun to progress to fibrosis, compared to those who solely have steatosis. Larger scale studies are clearly required to elucidate this further.

Very-low calorie formula diets or gastric banding has been used to promote more significant or rapid weight

loss to reduce hepatic fat content^[7-10]. Andersen *et al* placed morbidly obese subjects on a very low calorie liquid diet (388 kcal/d), resulting in a weight loss of 34 kg, and significant reduction in hepatic fat^[7]. Similarly, type-2 diabetic patients on very low fat (3%) liquid diets for 3-12 wk, lost 8 kg of body weight, with an 81% decrease in hepatic fat. Others have shown significant improvements in liver histology following large weight losses of over 30 kg^[8,9]. However, morbidly obese patients undergoing rapid weight reduction, may develop portal inflammation, fibrosis and hepatitis in addition to the decrease in hepatic fat content^[7,9]. Drug interventions have also been used to reduce hepatic fat and improve liver histology^[18-26]. Orlistat and metformin in combination with diet, can reduce in both weight and hepatic fat^[18,19], while metformin alone reduces body fat content (specifically subcutaneous AT), but has no effect on hepatic fat^[20]. Reductions in hepatic fat have been observed following treatment with pioglitazone^[21,22], rosiglitazone^[20,23-25] and pantethine^[26], despite the increases in body weight associated with glitazone therapy.

Only a few studies have compared changes in hepatic fat with changes in regional AT depots. Tiikkainen *et al* using diet restriction to reduce hepatic fat found that AT was lost from both subcutaneous and IAAT^[11]. However, unlike the present study they found no correlation between changes in liver fat and the changes in subcutaneous and IAAT. Carey *et al*, using rosiglitazone found an increase in subcutaneous AT, with no change in IAAT^[24]. Osono *et al* found decreased liver fat with pantethine, accompanied by a decrease in IAAT and an increase in subcutaneous AT^[26]. It is clear from the differences between these studies that diet and drug interventions may result in quite different mechanisms for the clearance of fat from the liver and reduction in AT volume. We found significant correlations between improvements in hepatic fat and reductions in both subcutaneous abdominal and IAAT. There were also strong correlations between decreased IAAT and decreased AST and ALT. However, regression analyses suggested that only reduction in IAAT could predict improvements in liver function and hepatic fat. Weight loss from other depots may well have other benefits, but not directly on hepatic function. A correlation was found between reduction in subcutaneous abdominal AT and S-IMCL, which may have implications for improving insulin sensitivity, since insulin resistant individuals have elevated IMCL^[33-35].

Several studies have looked at the effect of dietary restriction on AT content and distribution. A review of intervention strategies to reduce AT, suggests that most dietary interventions report a preferential loss of IAAT^[36]. Most papers included used a greater degree of calorie restriction than the present study and generally used females^[36]. In a study comparable to our own, Ross *et al* placed male subjects on a 700 kcal/d calorie restriction for 12 wk^[37]. They reported reductions of 0.7 kg and 0.8 kg from the abdominal subcutaneous and IAAT depots respectively^[37]. In our study, we found that similar proportions of AT were lost from both IAAT and subcutaneous abdominal depots, suggesting modest weight loss, achieved gradually, results in a non-selective loss of

AT from both IAAT and subcutaneous abdominal depots. A significant reduction in T-IMCL, but not in S-IMCL, was observed. Previous studies have shown decreases in S-IMCL in response to large weight loss (24%)^[38], but not to smaller reductions (8%-9%)^[10,11,38]. The tibialis may be more sensitive to weight change than the soleus. Indeed, T-IMCL may be more sensitive to catabolic lipid metabolism than the soleus^[39], which may explain its more significant response to intervention in this and previous studies^[40-43].

Both serum AST and ALT were reduced following weight loss, though only AST reached significance. Most studies looking at the relationship between liver enzymes and adiposity have focused on ALT, since elevated ALT is associated with obesity and insulin resistance^[44]. The lack of significance in the reduction in ALT observed in the present study could be explained by the suggested insensitivity of ALT to detect low levels of hepatic fat^[45]. Alternatively, although patients were referred to the study with elevated LFTs, the levels had improved somewhat by the time of inclusion in the study. Therefore despite ultrasound and subsequently MRS showing elevated liver fat, baseline LFT values in several subjects, although elevated, were not significantly outside the normal range. A significant decrease in LFTs within a 'normal' range may be difficult to achieve, this also indicates that normal LFTs are not necessarily a good indicator of liver fat content. Huang *et al* found no change in either AST or ALT following a similar study of nutritional counselling, despite improvements in liver histology^[12]. Interestingly, when their subjects were subdivided into responders and non-responders, the former showed a significant reduction in both ALT and AST^[12]. We observed similar effects, subjects showing reduced IHCL also showed a significant reduction in ALT. However, regression analyses suggest that changes in AST and ALT are only associated with modulation of IAAT during weight loss. Clearly, any relationship between hepatic steatosis and LFTs must include potential effects of other fat depots, especially IAAT. Several of the results in this study approached significance or were not significant, despite many suggesting a trend of 'improvement'. This is likely due to the small number of subjects ($n = 10$) included in this study, therefore type-2 statistical errors must be considered as confounding factors.

In summary, we have shown that modest weight loss achieved through UK clinically-implemented nutritional counselling regimens can result in worthwhile changes in regional adiposity, improvements in liver function, and reductions in both hepatic and tibialis muscle fat content. This study used clinically available, standard nutritional regimens as such, and our results are representative of clinical practice for NAFLD patients within the UK. Although there were some individual decreases in hepatic fat content, a more significant weight loss may be required to ensure consistent changes.

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CLINICAL RESEARCH

Adult-to-adult right lobe living donor liver transplantation: Comparison of endoscopic retrograde cholangiography with standard T2-weighted magnetic resonance cholangiography for evaluation of donor biliary anatomy

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CONCLUSION: Pretransplant ERC is safe and superior over standard MRC for detection of biliary variations that occur with a high frequency. However, precise knowledge of biliary variants did not reduce the incidence of postoperative biliary complications.

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Key words: Living donor liver transplantation; Donors biliary tree; Endoscopic retrograde cholangiography; Magnetic resonance cholangiography

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Abstract

AIM: To compare the value of endoscopic retrograde cholangiography (ERC) and standard T2-weighted magnetic resonance cholangiography (MRC) in the evaluation process as adult-to-adult right lobe living donor liver transplantation (LDLTx) demands a successful outcome, and exact knowledge of the biliary tree is implicated to avoid biliary complications, postoperatively.

METHODS: After starting the LDLTx program, 18 liver transplant candidates were selected for LDLTx by a stepwise evaluation process. ERC and standard T2-weighted MRC were performed to evaluate the biliary system of the donor liver. The anatomical findings of ERC and MRC mapping were compared using the Ohkubo classification.

RESULTS: ERC allowed mapping of the whole biliary system in 15/15 (100%) cases, including 14/15 (93.3%) with biliary variants while routine MRC was only accurate in 2/13 (15.4%) cases. MRC was limited in depicting the biliary system proximal of the hepatic bifurcation. Postoperative biliary complications occurred in 2 donors and 8 recipients. Biliary complications were associated with Ohkubo type C, E or G in 6/8 recipients, and 2/3 recipients with biliary leak received a graft with multiple (≥ 2) bile ducts.

INTRODUCTION

Over the past decade, a critical shortage of cadaveric organs for adults in need of liver transplants has developed. The current mortality for patients awaiting liver transplantation (LTx) ranges from 20% to 30%. During this time, the waiting period for LTx and the mortality among patients on waiting lists have increased by a factor of more than 10 while the donor pool has expanded only marginally^[1]. The use of adult-to-adult right lobe living donor liver transplantation (LDLTx) provides an alternative technique to reduce the waiting list mortality^[2-4]. Hereby, a transplant candidate gains survival time and quality of life while a proven healthy individual undergoes liver resection for living donation of right or left lobe. The greatest risk in LDLTx is the death of the donor which is estimated to range between 0.1% to 0.5%^[5-7]. Furthermore, a potentially uneventful outcome of the recipient is a psychological threat for the donor. Thus, healthy donors might undergo an extended invasive diagnostic during evaluation. Also, complications are relatively common in living liver donors^[8]. Therefore, donor safety has the highest priority when LDLTx is performed^[9], and selection

criteria and management of living donors requires continuous refinement^[10]. Finally, potential advantages in recipients must be faced against any potential risk of morbidity and mortality in the living donor.

Bile duct leaks and stenosis impact morbidity and mortality in the early and late phase after LTx^[11]. After LDLTx, only few studies focused on anatomical variations of the biliary tree, which is the rule rather than the exception in liver surgery^[12]. The reported incidences of absent hepatic duct are 26% for the right and 2% for the left side^[12]. In LDLTx, anatomic variations of intrahepatic bile ducts can complicate both, the donor and the recipient operation^[13]. Full hepatic lobectomy is required for adult-to-adult LDLTx, and the postoperative risk is greater after right lobe resection^[14]. Preoperative delineation of the biliary system appears important to achieve a successful outcome^[13].

Management of bile duct variations in LDLTx is technically demanding and lacking awareness of biliary variation increases the risk of postoperative complications^[15,16]. The biliary anatomy is mainly evaluated by endoscopic retrograde cholangiography (ERC), magnetic resonance cholangiography (MRC) or intraoperative cholangiography. The last might cause technical complications or results in oversight of biliary anomalies with consecutive biliary complications^[17]. Hitherto, about half of the LDLTx programs perform ERC for the evaluation of donor bile-duct anatomy, and about one third of the programs use MRC^[8]. Therefore, we compared the value of ERC and standard MRC in living donors prior to LTx.

MATERIALS AND METHODS

Subjects

Between March 1st, 2000 and October 5, 2005, 18 liver transplant candidates were selected for LDLTx by a stepwise evaluation process including identification of potential candidacy, assessment of clinical status and risk factors, assessment of liver function and anatomy, and final measures^[18].

Mean age of 18 donors (11 female, 7 male) was 50.4 ± 9.7 years, and 47 ± 9.2 years in 18 recipients (12 male, 6 female). Seven donors had genetic and 11 emotional relation to their recipient. Indications for LTx were hepatitis B or hepatitis C related cirrhosis (*n* = 6), post alcoholic cirrhosis (*n* = 4), hepatic metastasis of neuroendocrine tumor (*n* = 2), primary biliary cirrhosis (*n* = 1), primary sclerosing cholangitis (*n* = 1), cryptogenic cirrhosis (*n* = 1), bile duct cysts (*n* = 1), hepatic metastasis of gastrointestinal stroma tumor (*n* = 1), and echinococcus multilocularis hydatitis with post alcoholic cirrhosis (*n* = 1). Patients with cirrhosis were staged Child A (*n* = 3), B (*n* = 6), and C (*n* = 5) according to the Child-Pugh-Turcotte classification.

Evaluation of biliary anatomy

Biliary anatomy was evaluated by standard MRC and ERC. Standard MRC technique was provided by a radiologist in daily routine with Half-Fourier Spin-Echo in single shot technique and by Turbo-Spin-Echo with multiplanar

reconstruction in T2-weighted images. Standard ERC was performed by one experienced hepato-gastroenterologist. Patients received midazolam, propofol, and butylscopolaminbromide immediately before ERC. Serum amylase and lipase were controlled before and after ERC. Additionally, biliary anatomy was assessed by graft preparation and intraoperative cholangiography.

The biliary anatomy of MRC and ERC films were classified retrospectively according to Ohkubo^[12] by the specialists in radiology, hepato-gastroenterology, and transplantation surgery at our institution (Table 1).

RESULTS

Evaluation of donor biliary anatomy

ERC did not cause procedure-related complications. ERC (MRC) images were completely available in 15 (13) donors for retrospective analysis (Table 1). ERC allowed mapping of the whole biliary system in 15/15 cases (100%), while routine MRC was only accurate in 2/13 cases (15.4%). MRC was limited to depict the biliary anatomy only up to the first hepatic branches (Figure 1, Figure 2, Figure 3). Interestingly, 14/15 (93.3%) donors had biliary variants detected by ERC. Biliary anatomical variants classified according to Ohkubo *et al*^[12] are depicted in Table 1. Donor livers were classified type A (*n* = 2), B (*n* = 2), C (*n* = 4), E (*n* = 5), G (*n* = 1), and K (*n* = 1). Right hepatic duct (RHD) was present in 2 (13.3%) and absent in 12 (80.0%) donor livers. Left hepatic duct (LHD) was absent in one donor liver.

The biliary anatomy determined by preoperative ERC was identical to the intraoperative findings (Table 1). Seven of 18 (38.9%) grafts had one, 7/18 (38.9%) two, and 4/18 (22.2%) three bile ducts. In total, 11/18 (61.1%) right lobe grafts presented with two or more bile ducts.

Biliary complications

Postoperatively, 2/18 donors developed biliary complications (11.1%) including one biliary leak (Ohkubo type E, 2 bile ducts) and one bile duct stenosis (Ohkubo type C, one bile duct). Both complications occurred in livers with absent RHD. The biliary leak was located at the cutting surface and resolved without intervention, but the T-drain was kept for a longer period. Bile duct stenosis was successfully treated by endoscopic stenting. Eight of 18 (44.4%) recipients developed biliary complications (Table 1) including 3 biliary leakages, one bilioma, one bile duct necrosis, and 3 bile duct stenoses. Biliary leakages were associated with Ohkubo type G (*n* = 1) or not classified (*n* = 2). One leakage was related to stent dislocation. This graft had 3 bile ducts reconstructed by hepaticojejunostomy (HJS) with insertion of transhepatic stents. Two leakages were located at the cutting surface. A segment 6 necrosis occurred in one recipient who received an Ohkubo type C graft with one bile duct that was reconstructed by HJS and placement of a transhepatic stent. This patient also developed intrahepatic bilioma. Segmental necrosis might be related to transection of the arterial supply. One recipient developed bile duct necrosis located at the T-tube insertion side. Bile duct stenosis occurred in 3 recipients. One of these patients had received an Ohkubo type E graft including two bile

Table 1 Preoperative MRC, ERC, Ohkubo classification^[12] and intraoperative findings in donors, biliary reconstruction of the graft and postoperative biliary complications of recipients

Donor					Recipient			
No.	MRC	ERC	Ohkubo classification	Intraoperative findings	Biliary complications	Biliary reconstruction	Stents	Biliary complication
1	NA	NA	NA	3 BD	None	HJS	2 THS	Leak due to dislocated stent
2	Suspicion of early branches to SV and VI from RHD	SV drains into RHD, branch of SV drains into LHD	Type K	3 BD	None	2 D/D	T-drain	None
3	SV drains into LHD	SV and VI drain separately into LHD	Type C	2 BD	None	2 D/D	2 THS	None
4	Suspicion of doubled RHD	2 separate RHD	Type E	2 BD	Leak at cutting surface	2 D/D	2 THS	None
5	No variation	SVI drains separately into CHD	Type G	2 BD	None	2 D/D	2 THS	Leak at cutting surface
6	Early division of CHD	Early division of CHD	Type A	2 BD	None	2 D/D	T-drain	None
7	NA	NA	NA	1 BD	None	1 D/D	T-drain	None
8	No variation	SVII drains into LHD, SIVa drains into RHD	Type E Type K	1 BD	None	1 D/D	T-drain	None
9	No variation	Early division of RHD, SV drains directly into CHD	Type C	1 BD	BD stenosis	HJS	1 THS	SVI necrosis, intrahepatic bilioma
10	SVI drains directly into CHD	SVI and VII drains directly into CHD	Type E	2 BD	None	2 D/D	T-drain	BD stenosis 2 years after LDLTX
11	Doubled RHD	2 separate RHD	Type E	2 BD	None	2 D/D	T-drain	BD necrosis
12	NA	NA	NA	1 BD	None	1 D/D	1 THS	Leak at cutting surface
13	Not evaluable because of artifacts	No variation	Type A	1 BD	None	1 D/D	1 THS	None
14	Suspicion of early division of RHD	Trifurcation of RHD to SV, VI, and VIII	Type B	3 BD	None	3 D/D	1 THS	None
15	Suspicion of early division of RHD	Trifurcation of RHD to SV, VI, and VIII	Type B	3 BD	None	3 D/D	None	None
16	No variation	2 separate RHD	Type E	2 BD	None	HJS	None	BD stenosis 3 mo after LDLTX
17	NA	Early division of RHD	Type C	1 BD	None	HJS	1 THS	BD stenosis 6 mo after LDLTX
18	NA	Early division of RHD	Type C	1 BD	None	1 D/D	T-drain	None

NA: Not available; BD: Bile duct; HJS: Hepaticojunostomy; THS: Transhepatic stent; S: Segment; RHD: Right hepatic duct; LHD: Left hepatic duct; D/D: Duct-to-duct; CHD: Common hepatic duct; LDLTX: Living donor liver transplantation.

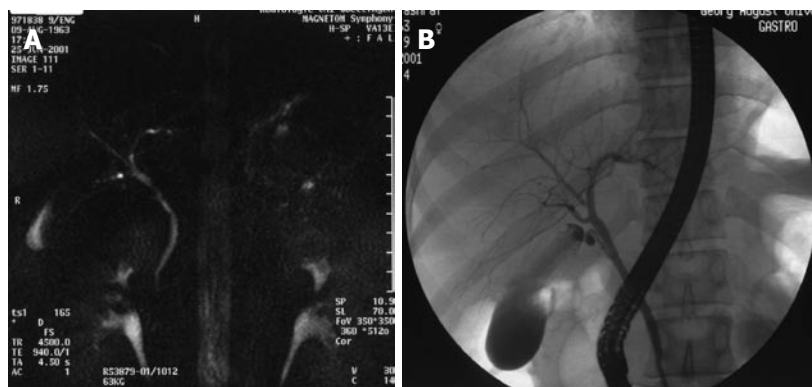


Figure 1 Preoperative MRC and ERC of donor No.11 with RHD. A: MRC, doubled; B: ERC, two separate.

ducts that were reconstructed duct-to-duct with insertion of a T-drain. Bile duct stenosis developed two years after transplantation and was successfully treated by endoscopic stenting. A second recipient also received an Ohkubo type E graft with two bile ducts reconstructed

by hepaticojunostomy. Bile duct stenosis occurred 3 mo after transplantation and was successfully treated by placement of a Yamakawa prosthesis. A third patient with an Ohkubo type C graft and one bile duct developed bile duct stenosis 6 mo after transplantation that was also

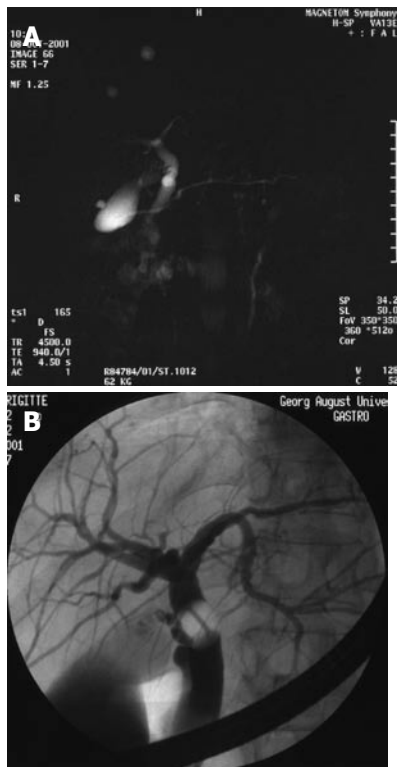


Figure 2 Preoperative MRC and ERC of donor No. 14 with RHD. **A:** MRC, suspicion of early division; **B:** ERC, trifurcation of RHD to SV, VI, and VIII.

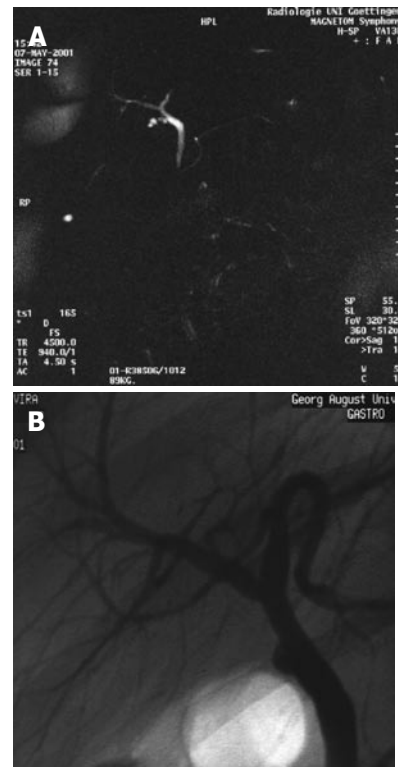


Figure 3 Preoperative MRC and ERC of donor No. 8. **A:** MRC, no variation; **B:** ERC, SVII drains into LHD, SIVA drains into RHD.

successfully treated by a Yamakawa prosthesis.

In summary, preoperative ERC detected biliary variants in 93.3% of donors. MRC confirmed ERC findings in only 15.4%, and was restricted in depicting the biliary anatomy above the hepatic bifurcation. Biliary complications occurred in 2 donors and 8 recipients. Ohkubo type C, E or G grafts were associated with biliary complications in 6/8 (75%) recipients. Two of 3 recipients with biliary leakage received a graft with multiple (≥ 2) bile ducts.

DISCUSSION

Determination of biliary anatomy in LDLTx can be performed preoperatively by ERC, MRC, contrast-enhanced CT-cholangiography, and intraoperative cholangiography. MRC is less invasive than ERC that can cause pancreatitis as a known complication. In a meta-analysis of 15 prospective trials using endoscopic retrograde cholangio-pancreaticography (ERCP), 14 risk factors of ERCP-associated pancreatitis have been identified involving nine related to the endoscopic technique (e.g. precut sphincterotomy) with a relative risk of 2.2 to 4.09^[19]. In our series, we used ERC only diagnostically without intervention or pancreatic injection. ERC was well tolerated and none of the patients developed pancreatitis or other procedure related complications. In contrast to standard routine MRC, ERC allowed complete mapping of the biliary system. Preoperative ERC detected variations of biliary anatomy in 93.3% of donors that were confirmed by our intraoperative findings.

The inaccuracy of standard routine MRC in our series might be explained by the imaging slice thickness and artifacts. Recently, new modified MRC techniques such as volumetric mangafodipir trisodium enhanced MRC, heavily T2-weighted thin section MRCP, and gadobenate

dimeglumine enhanced MRC have been developed^[20-24]. Sensitivity of heavily T2-weighted axial/coronal thin section MRCP for normal biliary anatomy was 89.5% and 71.4% for variants in LDLTx. The accuracy in depicting biliary anatomy was 84.6%^[22]. In another series, the sensitivity, specificity, positive predictive, and negative predictive value of preoperative heavily T2-weighted radial slab MRC was 92%, 100%, 100%, and 94%^[24]. Mangafodipir trisodium enhanced 3D MRC in combination with conventional T2-weighted MRC depicted the intrahepatic biliary anatomy correctly in 94% of living liver donors. Especially right duct variants were more accurately depicted with mangafodipir trisodium enhanced 3D MRC compared to conventional T2-weighted MRC^[21]. Another new technique of mapping the biliary anatomy is multidetector computed topography cholangiography (MDCT-CA) with 1 mm collimation^[16]. This method depicted anatomical biliary variations up to the fourth level of intrahepatic branches. Superiority of MDCT-CA over conventional MRC is explained by better spatial resolution using 1 mm instead of 4 mm slice thickness. Also, conventional MRC is susceptible for artifacts in T2-weighted images. Therefore, the use T1-weighted images is recommended in combination with Gd-EOB-DTPA or Gd-BOPTA, both biliary excreted contrast-agents^[25,26]. Also, Gd-BOPTA-MRC can be combined with CE-MRA without injection of a second contrast agent^[16,26]. Gd-BOPTA-enhanced MRC is superior over T2-weighted MRC sequences in mapping the biliary tree, but spatial resolution remains moderate^[25,26]. In principal, both imaging modalities can be performed as an all-in-one-protocol for evaluation of liver parenchyma, arterial, venous, and biliary anatomy. However, MDCT-CA harbors potential risks like X-ray exposure and allergic reaction to contrast medium. Both risk factors can be

avoided with Gd-BOPTA T1-weighted MRC^[26]. However, the role of the above-mentioned procedures needs to be defined for evaluation of biliary anatomy in living liver donors in the near future. At present these new techniques have not been compared to preoperative ERC yet.

In our series, biliary complications occurred in 2/18 (11.1%) donors. In larger series, incidence of biliary complications ranged from 4% to 13%^[27-31]. Incidence of biliary leaks at the cutting surface is approximately 5% in living donors^[32-36] and was 5.6% (1/18 donors) in our series. Notable, donors with absent RHD (Ohkubo type C and G) grafts are at risk to develop biliary leakage or obstruction^[12]. Both biliary complications in our donors occurred in livers with absent RHD (Ohkubo type C and E). In recipients, biliary complications are the Achilles' heel of all segmental LTx^[9]. Incidence of biliary complications in LDLTx recipients is 15%-40%^[8,9,15]. In our series, 8/16 (44.4%) recipients had complications despite exact preoperative determination of biliary variants by ERC. Nevertheless, anatomic variations of the biliary tract are common but may not contraindicate donation^[13,37]. The risks of biliary complications increase with the number of bile duct openings in the graft^[38]. Rogiers *et al*^[9] focused on two complex biliary variations: (1) Ramus posteriomedialis (RPM) drains into the common hepatic duct (CHD), ramus lateralis (RL) merges with segment (S) II, and drain together into the left hepatic duct (LHD), and (2) RPM drains into CHD; S I, S II, S III, and S IV drain separately or together into RL. These two biliary variations cease splitting of the liver in selected cases. Recently, a case was published in which an anatomic biliary variation was seen as a contraindication for right lobe LDLTx^[38].

In conclusion, biliary anatomy in living liver donors is highly variable, but does not exclude from donation. ERC is superior to the standard MRC technique for detailed preoperative mapping of intra- and extrahepatic bile ducts, but does not reduce the incidence of postoperative biliary complications.

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CLINICAL RESEARCH

Fatty liver, carotid disease and gallstones: A study of age-related associations

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Abstract

AIM: To evaluate carotid intima-media thickening (IMT) and plaques, gallstone disease (GD) and fatty liver (FL) as a function of age.

METHODS: In 449 subjects, FL and carotid disease were assessed ultrasonographically. In a subgroup of 65/449 patients with non-alcoholic fatty liver disease (NAFLD), carotid disease, GD and associated factors were determined.

RESULTS: FL of unspecified etiology was more common in younger and GD in older individuals. FL subjects had an increased prevalence of IMT and a decreased prevalence of plaques and manifested carotid disease earlier. Plaques were more common in subjects with GD. Age was an independent predictor of carotid disease outcome and FL was a protective factor for plaques. In NAFLD, there was an inverse correlation between body weight and age and the latter independently predicted carotid findings.

CONCLUSION: Cardiovascular risk in patients with FL and NAFLD needs to be assessed as a function of age and body weight.

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Key words: Atherosclerosis; Carotid; Gallstones; Intima-media thickening; Fatty liver

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INTRODUCTION

Atherothrombosis (AT), fatty liver (FL) and gallstone disease (GD) represent a clinically heterogeneous group of disorders which commonly occur in the general population and impose a heavy economic burden for direct and indirect health expenditures^[1-4]. Given that insulin resistance is an acknowledged risk factor for their development^[5-7], it is expected that AT, non-alcoholic fatty liver disease (NAFLD), one of the most common etiologies of fatty liver, and GD might often affect the same individuals. Several studies have suggested a link between NAFLD and endothelial dysfunction, carotid intima-media thickening (IMT) and carotid plaques^[8-10]. However, the development of AT is a complex process involving endothelial dysfunction and IMT as early lesions. IMT, in particular, does not represent definite AT disease but rather it is a marker for its development^[10]. In addition, some previous studies may not be representative of NAFLD due to enrollment of only male patients in a narrow range of body mass index (BMI) and age^[9,11]; therefore, the occurrence of increased cardiovascular risk in NAFLD remains a controversial issue^[12,13].

NAFLD patients might theoretically be prone to GD *via* impaired gallbladder motility and increased bile lithogenicity. Both physiopathologic mechanisms occur in type 2 diabetes mellitus (T2DM) and obesity, which are often associated with NAFLD^[7]. However, the NAFLD-GD association remains uncertain given that it has been reported in some studies but rejected in others^[14-17]. Furthermore, the association between AT and GD has been reported^[18-20], but some authors argue this association might be spurious and influenced by confounders^[21]. Although they share a common pathogenetic mechanism, AT, GD and NAFLD occur to a different extent in various age groups. For instance, increasing age is a risk factor for the development of AT and GD^[1,4] but a protective factor for NAFLD^[22]. Given that no previous studies have evaluated simultaneously the occurrence of AT, GD and FL, we evaluated the patterns of age distribution of FL of

unspecified etiology and its association with carotid disease and GD in either gender across a wide range of age-groups. In addition, we also assessed the independent predictors of carotid disease in a subgroup of patients with definite NAFLD.

MATERIALS AND METHODS

Patients

The electronic database of ultrasound reports for in- and out-patients examined at Modena City Hospital (Operating Unit Internal Medicine and Gastroenterology, to whom one of the authors belonged at the time when data were collected) was searched. Criteria for inclusion in the study were to have undergone both liver and carotid ultrasonographic assessment in the same day or, in any case, not later than 12 mo apart. Focal liver lesions, ascites or other ultrasonographic stigmata of portal hypertension (such as ascites, splenomegaly, presence of patent umbilical vein, varices in the hepato-gastric ligament or spontaneous spleno-renal shunts) were criteria for exclusion. Approximately 1200 liver ultrasound examinations per year were recorded in the 1993-2005 period of time; out of them, all 449 subjects fulfilling the enrollment criteria were selected.

In 384 of 449 cases, information on gender, age, presence/absence of FL and evaluation of carotid morphology was available. No information on being in- or out-patient, final diagnosis, etiology of FL or anthropometric and biochemical data was included in the software of the electronic database. Sixty-five of 449 subjects were part of the series recruited into the POLISTENA study and thus their bright livers were due to NAFLD. In this sub-group, in addition to the previous parameters including liver and carotid US evaluation, a complete history, anthropometric parameters and metabolic data were also available as a part of the protocol of the POLISTENA study^[23,24].

In the entire population of 449 subjects, we evaluated the prevalence of FL of unspecified etiology and its relationship with the US carotid findings and GD. In the subgroup of 65 NAFLD patients, the prevalences of carotid alterations, GD and the associated factors were determined.

Parameters evaluated in the whole population

Fatty liver of unspecified etiology: The presence of FL was evaluated through ultrasound scanning with a 3.5 MHz commercially available transducer and defined by the main criterion of "bright" liver as described elsewhere^[25]. Additional diagnostic criteria considered were vessel blurring and posterior attenuation of the ultrasound beam. Ultrasound was performed by trained physicians who were unaware of the results of the carotid evaluation. The evaluation of presence/absence of FL among the various operators was evaluated using Chi-square test and no significant difference was found.

Gallstone disease: GD was defined by the presence of one or more echogenic, distal acoustic shadowing, possibly moveable structures in the gallbladder or empty gallbladder fossa in a subjects with a history of cholecystectomy^[7].

Carotid intima-media thickening and plaque: Carotid ultrasound evaluation was performed by a single trained operator who was blind to the clinical features of patients.

Repeated measurements on the same subject gave coefficients of variation within 5%. The imaging unit was a commercially available machine (AU 600, Hitachi Medical Co., Tokyo, Japan) equipped with a 5-MHz linear array commercially available transducer. The pulsed Doppler frequency was 4.0 MHz. The imaging protocol^[26] involved scanning along the entire course of the cervical carotid artery from the supraclavicular notch cephalad to the angle of the mandible. Examination of the right and left common and internal carotid arteries was also performed. The maximum wall thickness observed for either side was taken into consideration. Findings were classified as: (1) normal (intima < 1 mm); (2) intimal thickening (intima-media measuring ≥ 1 mm and < 1.3 mm); and (3) plaque (focal widening of the vessel wall relative to adjacent segments ≥ 1.3 mm) or complicated plaque (any plaque narrowing > 70% of the lumen).

Parameters evaluated in the subset of patients with NAFLD

Diagnosis of NAFLD: The diagnosis of NAFLD in the subgroup of 65 patients enrolled in the POLISTENA study was based on: (1) absent-to-low alcohol consumption (≤ 30 g alcohol daily for men and ≤ 20 g for women); (2) evidence of FL at ultrasound scanning as detailed above; and (3) absence of alternative etiologies of chronic liver disease, notably viral, autoimmune, thyroid, drug-induced, hemodynamic and genetic-metabolic (alpha1-antitrypsin, hemochromatosis, Wilson disease). In addition to abdominal ultrasonography and ultrasound carotid scanning, all patients underwent a questionnaire, physical examination and blood sampling for biochemical analysis. In 23 of 65 subjects, liver biopsy was clinically indicated on the grounds of hepatomegaly and/or particularly altered liver function tests and was performed under ultrasound guidance.

Questionnaire: The questionnaire investigated family and personal history, including concurrent diseases, previous surgery, past and current use of medications, possible contacts with toxic agents. Alcohol intake, smoking habits and physical activity were also recorded. Alcohol consumption was assessed through separate interviews with the patient, the referring physician, and family members. Subjects were classified as never smokers (never smoked currently or in the past 5 years) or current smokers. The latter group comprised those smokers of as much as one cigarette a day and also included those ex-smokers who had quit tobacco since less than 5 years. Physical activity was defined as any exercise, such as jogging, swimming, attending a gymnastics course, cycling, playing tennis, dancing or heavy gardening. Based on their leisure time physical activity, subjects were classified as sedentary (those exercising less than 1 h per week) or non-sedentary (all the others).

General physical examination: It was performed by one of the senior authors. Anthropometric parameters and blood pressure measurements were performed by a trained nurse. Waist girth was measured at the smallest circumference between the ribs and the iliac crest and hip at the maximum circumference between the iliac crest and the crotch. Waist-hip ratio (WHR) was calculated as waist circumference/hip circumference.

Laboratory tests: Laboratory evaluation included renal and liver function tests, blood cell count, total and fractionated proteins, parameters of iron metabolism (serum iron, transferrin and ferritin), lipid profile (total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, apo-A, apo-B), fasting glucose, fasting insulin, and uric acid. Serum concentrations of copper, ceruloplasmin and alpha1-antitrypsin were performed to exclude Wilson disease and alpha1-antitrypsin deficiency. Non-organ-specific autoantibodies were also evaluated. In all patients, the presence of viral serologic markers for B and C infection was assessed by standard methods. Insulin resistance was calculated according to the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) index computed through the formula: fasting serum insulin ($\mu\text{U/mL}$) \times fasting serum glucose (mmol/L)/22.5^[27]. The diagnosis of impaired glucose tolerance and diabetes was based on the American Diabetes Association criteria^[28]. Subjects with impaired glucose tolerance included individuals with fasting glucose levels ≥ 6.1 mmol/L but < 7 mmol/L and with a 2-h post-load glucose ≥ 140 mg/dL (7.8 mmol/L) and < 200 mg/dL (11.1 mmol/L). Diabetes prevalence was based on the number of individuals with fasting plasma glucose ≥ 126 mg/dL (7 mmol/L) checked twice or having a past history of diabetes^[28]. Body mass index (BMI) was calculated with the formula: mass (kg)/height (m)² and obesity was defined by BMI ≥ 30 kg/m². Normal limits for blood lipids were defined accordingly to the third report of the National Cholesterol Education Program (NCEP). Hypercholesterolemia was defined as serum cholesterol ≥ 5.10 mmol/L, hypertriglyceridemia as serum triglycerides ≥ 1.65 mmol/L^[29]. Hypertension was diagnosed if patients had a past history of hypertension, were taking antihypertensive drugs or had a blood pressure $\geq 140/90$ mmHg.

Liver biopsy: Sections were stained with H&E; silver stain for reticulin, Sirius red for collagen, periodic acid Schiff for glycogen, periodic Schiff-diastase for glycoproteins, and Perl's for iron. Liver biopsy specimens were considered to be adequate if 6 or more portal tracts were included. Evaluation of steatosis and fibrosis was performed following the criteria developed by Brunt *et al*^[30]. A diagnosis of NASH required the following features: steatosis, parenchymal inflammation and ballooning degeneration of hepatocytes with or without fibrosis and Mallory bodies^[31]. Informed written consent was obtained from all participating individuals before taking blood samples and prior to liver biopsy. The study was performed in agreement with the Declaration of Helsinki.

Statistical analysis

Data were expressed as mean \pm SE for variables normally distributed and as median (25th-75th percentile) for those not normally distributed. Normalization of age was achieved through square transformation. The following tests were used as appropriate: Chi-square, Mann-Whitney, Oneway Scheffe's test, ANOVA, linear regression, binary logistic regression. Given that vascular carotid disease may manifest three different features (normal findings, IMT and plaques), we chose to adopt multinomial logistic regression which enables us to keep this distinction in

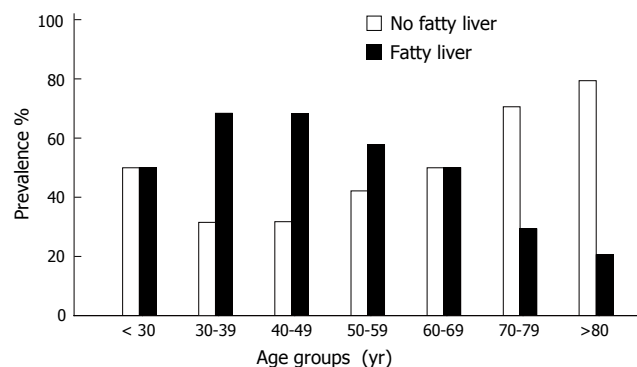


Figure 1 Distribution of ultrasonographically diagnosed fatty liver of unspecified etiology in the various age-groups of adulthood. The number of patients enrolled in each age-group is as follows: < 30: 8 cases; 30-39: 19 cases; 40-49: 44 cases; 50-59: 64 cases; 60-69: 98 cases; 70-79: 119 cases; and >80: 97 cases.

carotid ultrasonographic findings. The software used was SPSS Inc. Chicago, Illinois USA, release 14.0.

RESULTS

Data analysis in the whole population

Of the 449 subjects fulfilling enrollment criteria, 203 (45.2%) were men and 246 (54.8%) women; the median of the age was 69 years (56.5-78 years). Men were younger [median: 66 years (53-76 years)] than women [median: 72 years (59-81.25)] (Mann-Whitney test, $Z = -3.419$, $P = 0.001$).

Fatty liver of unspecified etiology

FL was present in 42.1% (189/449) of subjects, and the majority (102/189, 54.0%) were males ($\chi^2 = 10.104$, $P = 0.0014$). The distribution of FL per age-group is shown in Figure 1. The median age of the subjects with FL was 62 years (49-72 years) *versus* 75 years (65-81.75) in those without FL (Mann-Whitney test, $Z = -7.479$, $P = 7.52 \times 10^{-14}$). Logistic regression analysis using FL as the dependent variable and age and sex as predictors disclosed OR = -0.956, 95% CI = 0.943-0.969 ($P = 2.12 \times 10^{-10}$) for age and OR = -0.631, 95% CI = 0.422-0.942 ($P = 0.024$) for sex, demonstrating that FL is more common in younger male individuals.

Gallstone disease

The overall prevalence of GD was 32.5% (146/449), not significantly different in patients with FL *versus* those without FL (28.6% *vs* 35.2%; $\chi^2 = 2.315$, $P = 0.128$). Patients with GD were older than those without GD [73 years (61-82 years) *vs* 68 years (53-77 years); Mann-Whitney test, $Z = -3.497$, $P = 0.00047$] and GD was present more often in women than in men (38.6% *vs* 25.1%; $\chi^2 = 9.230$, $P = 0.002$). Logistic regression analysis using GD as the dependent variable confirmed that advanced age and female gender rather than steatosis predict the presence of GD (OR for age: 1.024, 95% CI: 1.009-1.039, $P = 0.002$; OR for female gender: 1.698, 95% CI = 1.118-2.578, $P = 0.013$). Fifty-four subjects had concurrent FL and GD. Compared with subjects with FL alone, these patients had more advanced median age [64 years (53-73 years) *vs* 60

Table 1 Ultrasonographic carotid findings in subjects with or without fatty liver of unspecified etiology

Carotid findings	Fatty liver		<i>P</i>
	Absent <i>n</i> (%)	Present <i>n</i> (%)	
Normal	19 (7.3)	39 (20.6)	$P = 4.65 \times 10^{-10}$
IMT	27 (10.4)	45 (23.8)	
Plaques	214 (82.3)	105 (55.6)	

χ^2 test.

Table 2 Median age and 25th-75th percentile of subjects with/without fatty liver of unspecified etiology (classified according to carotid findings)

Carotid findings	Fatty liver	
	Absent <i>n</i> (%)	Present <i>n</i> (%)
Normal	44 (31-52)	46 (34-58)
IMT	65 (51-71)	56 (48-62.5)
Plaques	76 (68.75-82)	68 (60.5-78)

Table 3 Carotid ultrasonographic findings in subjects with or without gallstone disease

Carotid findings	Gallstone disease		<i>P</i>
	Absent <i>n</i> (%)	Present <i>n</i> (%)	
Normal	48 (15.8)	10 (6.8)	$P = 0.015$
IMT	51 (16.8)	21 (14.4)	
Plaques	204 (67.3)	115 (78.8)	

χ^2 test.

years(48-71 years); Mann-Whitney test, $Z = -2.121$, $P = 0.034$], but there was no female gender prevalence (females 55.6% *vs* males 44.0%; $\chi^2 = 2.76$, $P = 0.097$).

Carotid ultrasonographic-Doppler evaluation

Fifty-eight of 449 subjects (12.9%) had normal carotid ultrasonographic findings. IMT was found in 16.0% (72/449) of subjects; the prevalence of atherosclerotic plaques was 71% (319/449). The distribution of carotid lesions per age-group is shown in Figure 2. All 8 subjects aged < 30 years had normal carotid findings (Figure 2). Carotid ultrasonographic-Doppler evaluation showed that median age was 45 years (33.75-55.25) for normal, 58 years (48.25-65) for IMT, 75 years (65-81) for plaques (Scheffe's Oneway on squared age $F = 134.118$; $P = 2.48 \times 10^{-46}$).

Relationship between FL of unspecified etiology, GD and carotid ultrasonographic-doppler findings

Table 1 summarises carotid findings as a function of presence/absence of FL. The frequency of subjects with IMT was more than two-fold in those with FL than in those without FL (23.8% *vs* 10.4 %). In contrast, the prevalence of plaques was decreased in those with FL of unspecified etiology than in those without it (55.6% *vs* 82.3%; $\chi^2 = 38.373$, $P = 4.65 \times 10^{-10}$).

Table 2 shows median age and 25th-75th percentile in subjects with/without FL as a function of carotid findings.

Table 4 Independent predictors of intima-media thickening (IMT) or plaques in fatty liver of unspecified etiology

Carotid findings		B	Standard error	<i>P</i>	Exp (B) (95% CI)
IMT	Intercept	-3.736	0.916	0.000	
	Male	-0.019	0.405	0.962	0.981 (0.443-2.169)
	Age	0.079	0.016	0.0000014	1.082 (1.048-1.117)
	Fatty liver	0.093	0.426	0.827	0.911 (0.395-2.101)
Plaques	Intercept	-7.879	1.050	0.000	
	Male	0.042	0.403	0.1719	1.719 (0.781-3.786)
	Age	0.166	0.018	2.27×10^{-20}	1.181 (1.040-1.223)
	Fatty liver	0.873	0.416	0.036	0.418 (0.185-0.945)

Multivariate multinomial logistic regression. Dependent variable: carotid findings (IMT or plaques). Reference category: normal carotid findings. Age, sex (forced entry terms), fatty liver and GD (forward entry term) were entered as covariates.

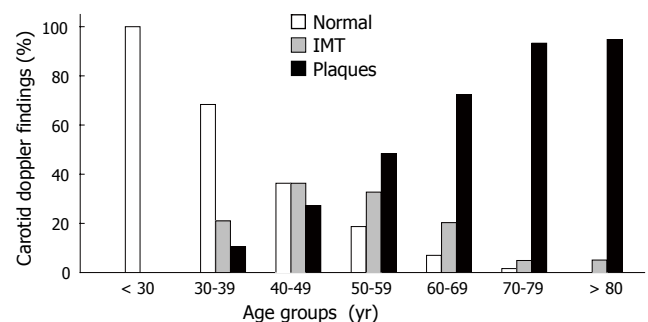


Figure 2 Distribution of carotid ultrasound findings in the various age-groups of adulthood. The number of patients enrolled in each age-group is the same as reported in Figure 1.

Following normalization obtained through square elevation, age was compared through ANOVA. Carotid findings ($F = 114.457$, $P = 8.47 \times 10^{-41}$) and FL ($F = 6.311$, $P = 0.012$) rather than gender (data not shown, $F = 1.529$, $P = 0.217$) were statistically different. Subjects with GD had a higher prevalence of plaques than those without plaques (78.8% *vs* 67.3%; $\chi^2 = 8.351$, $P = 0.015$) (Table 3). In order to determine those variables that were predictive of plaques or IMT, multinomial logistic regression was used. Carotid findings were the dependent variable (normal as the reference value) and age and gender as fixed covariates; factors were FL and GD (forward). As shown in Table 4, age turned out to be an independent predictor of ultrasonographic carotid outcome, OR for IMT being 1.082 (95% CI: 1.048-1.117, $P = 1.40 \times 10^{-6}$) and for plaques 1.181 (95% CI: 1.140-1.228, $P = 2.27 \times 10^{-20}$). The presence of FL was a protective factor for plaques (OR = 0.418, 95% CI: 0.185-0.945, $P = 0.036$) rather than for IMT (OR = 0.911, 95% CI: 0.443-2.169, $P = 0.827$).

Data analysis of the subset of patients with NAFLD

In 65 of 449 subjects belonging to the POLISTENA

Table 5 Historical, anthropometric and laboratory findings of subjects with NAFLD as a function of ultrasonographic carotid findings (mean \pm SE, 25th-75th percentile)

Variable	Normal (n° 19)	IMT (n° 30)	Plaques (n° 16)	P
Age (yr)	46.11 \pm 2.66	55.23 \pm 1.59	58.69 \pm 2.67	0.001
Gender (M/F)	10/9	13/17	8/8	NS
Smokers	8/17	10/30	6/16	NS
Physical exercise	5/17	11/30	6/16	NS
BMI (kg/m ²)	31.05 \pm 1.40	28.85 \pm 0.68	27.30 \pm 0.85	NS
Mass (kg)	87.21 (76.00-100.00)	79.9 (71.00-88.50)	74.28.50 (66.50-84.00)	0.026
Waist (cm)	102.53 \pm 2.73	96.81 \pm 2.02	95.31 \pm 2.19	NS
Hip (cm)	114.79 \pm 3.31	109.30 \pm 1.52	107.31 \pm 1.44	NS
W/H ratio	0.90 \pm 0.02	0.89 \pm 0.01	0.89 \pm 0.02	NS
Arterial pressure (mmHg)	D:80.0 (70.0-80.0) S:120.0 (120.0-130.0)	D:80.0 (80.0-80.0) S:130.0 (120.0-130.0)	D: 80.00 (80.00-87.5) S:1300 (120.0-145.0)	NS
Fasting glucose (mmol/L)	5.55 (4.75-6.54)	5.39 (5.05-5.83)	5.28 (4.99-5.65)	NS
Uric acid (μ mol/L)	345.29 \pm 21.76	333.52 \pm 14.11	315.88 \pm 18.23	NS
Total cholesterol (mmol/L)	5.37 \pm 0.26	4.15 \pm 0.21	5.91 \pm 0.18	NS
HDL Cholesterol (mmol/L)	1.20 \pm 0.06	1.25 \pm 0.06	1.21 \pm 0.06	NS
Triglycerides (mmol/L)	1.46 (0.99-2.05)	1.49 (1.18-1.98)	1.64 (1.49 - 2.00)	NS
AST (IU/L)	26.00 (23.00-35.00)	25.00 (21.00-31.50)	23.50 (19.50-29.50)	NS
ALT (IU/L)	53.00 (35.00-74.00)	39.00 (29.50-54.00)	36.00 (23.25-40.00)	NS
GGT (IU/L)	40.00 (29.00-71.00)	30.00 (21.00-71.00)	33.00 (24.25-49.25)	NS
Fasting insulin (pmol/L)	119.82 (86.81-145.65)	108.70 (79.28-164.88)	80.71 (60.62-109.77)	NS
Insulin 120 min post-load (pmol/L)	499.38 (269.78-1442.18)	767.72 (324.31-1379.39)	554.26 (383.50-770.45)	NS
APO A (mg/dL)	139.80 \pm 7.73	144.58 \pm 4.74	137.64 \pm 6.03	NS
APO B (mg/dL)	98.00 \pm 6.61	111.15 \pm 5.04	110.73 \pm 6.39	NS
Iron (μ mol/L)	16.34 \pm 1.04	16.58 \pm 0.89	15.99 \pm 1.39	NS
Ferritin (mg/dL)	122.68 \pm 23.64	129.00 \pm 17.95	175.36 \pm 30.22	NS
Transferrin saturation (%)	25.99 \pm 1.72	29.33 \pm 1.80	28.17 \pm 2.86	NS
Fibrinogen (g/L)	3.16 \pm 0.18	3.53 \pm 0.14	3.33 \pm 0.22	NS
HOMA-IR index	4.207 (2.936-5.941)	3.864 (2.708-5.869)	2.815 (2.256-3.861)	NS
Lp(a) (mg/dL)	35.00 (8.50-77.00)	12.00 (4.00-30.00)	9.00 (5.50-28.5)	NS

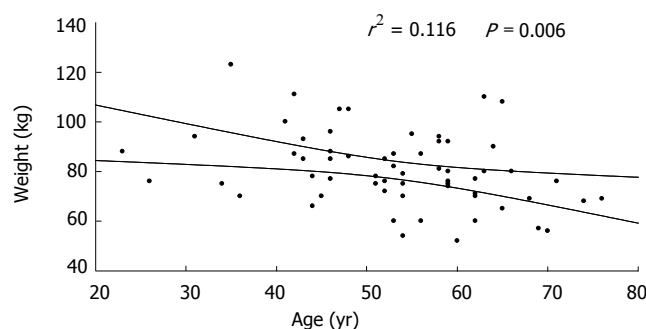
Significance of the comparisons evaluated through oneway Scheffe's test for variable normally distributed, and Kruskal Wallis for those not normally distributed.

study^[22,23], ultrasound carotid examination was also performed. These patients had an average age of 53.42 ± 1.38 years. Thirty-one of 65 (47.7%) were males with an average age of 48.06 ± 2.03 years, which was significantly lower than the age observed in women 58.29 ± 1.44 years ($t = -4.159$, $P = 0.00001$). The prevalence of components of the metabolic syndrome was as follows: hypercholesterolemia 72.3% (47/65); arterial hypertension 38.4% (25/65);

Table 6 NAFLD. Hepatic histological findings in NAFLD patients with normal carotid findings, intima-media thickening (IMT) and atherosclerotic carotid plaques¹

Hepatic histology	Carotid findings			p
	Normal n (%)	IMT n (%)	Plaques n (%)	
Inflammatory grade				
0	2 (29)	1 (10)	2 (33)	0.516 ²
1	3 (43)	5 (50)	2 (33)	
2	2 (29)	2 (20)	2 (33)	
3	0 (0)	2 (20)	0 (0)	
Fibrosis stage				
0	5 (71)	7 (70)	5 (83)	0.762 ²
1	1 (14)	1 (10)	1 (17)	
2	1 (14)	2 (20)	0	
3	0	0	0	
4	0	0	0	0.131 ³
Steatosis extent (%)	45.8 \pm 9.9	51.0 \pm 8.9	23.00 \pm 5.4	

¹23 of 65 NAFLD patients underwent liver biopsy; 7 had normal findings, 10 IMT, and 6 carotid plaques. ²Kruskal-Wallis test; ³One way analysis of variance.

**Figure 3** Inverse correlation between body weight and age in NAFLD patients.

obesity 36.9% (24/65); hypertriglyceridemia 33.85% (22/65); IGT 27.7% (18/65); and T2DM 21.5% (14/65). Out of 65 patients, 10 (15.4%) had GD. These GD patients were older than those without GD (59.00 ± 1.65 years *vs* 52.40 ± 1.56 ; $t = -2.91$, $P = 0.007$), with a similar proportion of males and females (9.68% *vs* 20.59%; Fisher's exact test, $P = 0.309$).

Patients with normal carotid findings were younger than those with IMT and with plaques (Oneway Scheffe's test, $F = 7.652$, $P = 0.001$). Individuals with plaques had lower body weight (Oneway Scheffe's test, $F = 3.878$, $P = 0.026$) and transferrin serum levels (Oneway Scheffe's test, $F = 4.491$, $P = 0.015$) than those with normal carotid findings (Table 5), indicating a significant inverse relationship ($r^2 = 0.116$; $P = 0.006$) between age and body weight (Figure 3). We also found a direct relationship between HOMA and BMI ($r^2 = 0.26$, $P = 0.000017$) rather than between HOMA and age. No relationship was found between histological variables and carotid ultrasonographic findings in the limited number of cases available (Table 6).

The distribution of carotid ultrasonographic findings in the various age-groups of adulthood is shown in Figure

Table 7 Independent predictors of intima-media thickening (IMT) or plaques in NAFLD subjects

Carotid findings		B	Standard error	P ¹	Exp (B) (95% CI)
IMT	Intercept	-0.955	2.648	0.718	
	Age	0.087	0.034	0.010	1.091 (1.021-1.165)
	BMI	-0.102	0.071	0.150	0.903 (0.785-1.038)
Plaques	Intercept	-0.913	3.628	0.801	
	Age	0.119	0.042	0.005	1.126 (1.037-1.223)
	BMI	-0.189	0.097	0.052	0.828 (0.684-1.002)

¹Multivariate multinomial logistic regression. Dependent variable: carotid findings (IMT or plaques). Reference category: normal carotid findings. Age, body mass (forced entry terms) and HOMA (forward entry term) were entered as covariates.

4. Variables associated with IMT and plaques (the reference being normal findings) were evaluated using univariate logistic multinomial regression, where age, BMI and HOMA were found to be significant independent predictors (data not shown). In the multivariate logistic multinomial regression (Table 7), only age turned out to be the significant independent predictor of carotid IMT (OR: 1.091, 95% CI: 1.021-1.165, $P = 0.010$) and plaques (OR: 1.126, 95% CI: 1.037-1.223, $P = 0.005$).

DISCUSSION

To the best of our knowledge, this is the first study which explores the prevalence and determinants of carotid disease, FL and gallstones. A major finding of this study was that distribution of FL per age-classes (Figure 1) peaked in the 30-49 years age-group and declined in the younger and more advanced age groups. This “inverted U” pattern resembles that reported for FL distribution in the general population in Japan^[14] and closely mirrors the curve of altered transaminase levels in USA^[32,33] and in Israel^[34]. Taken collectively, these data further support the theory that FL, most likely NAFLD, accounts for the vast majority of altered liver function tests (LFTs) in the Western world. We have no explanation as to why the prevalence of FL of unspecified etiology declined after the age of 49 years. Several hypotheses can be put forward. The phenomenon may reflect a simple decrease in risk factor for NAFLD, notably including obesity, as supported by the finding in the present study that body weight declines with age (Figure 3). An alternative explanation is that mortality might be selectively increased among those with FL of unspecified etiology. A recent study has shown that the presence of NAFLD is associated with an increased mortality as compared with the general population^[35]. A unifying explanation could be that only those NAFLD subjects who lose weight will survive till more advanced age. This hypothesis, though, remains speculative and needs support from prospective studies.

Relevant findings of this study are that subjects with FL of unspecified etiology had an increased prevalence of IMT and a decreased prevalence of plaques; in contrast, GD was associated with increased prevalence of plaques. Of interest, carotid disease was found to occur in

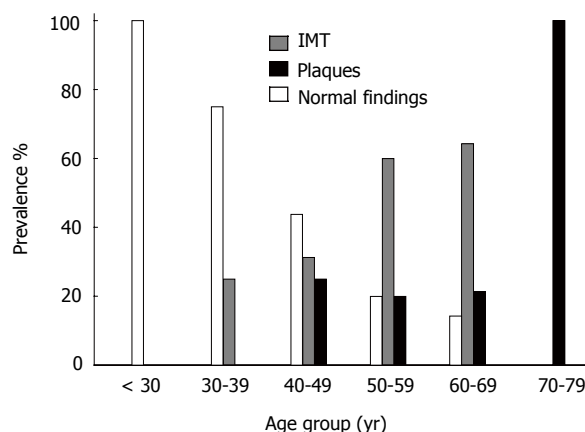


Figure 4 Distribution of carotid ultrasound findings in the various age-groups of adulthood with NAFLD. The number of patients enrolled in each age-group is as follows: < 30: 2 cases; 30-39: 4 cases; 40-49: 16 cases; 50-59: 25 cases; 60-69: 14 cases; and 70-79: 4 cases.

FL patients approximately 8 to 9 years earlier than those observed in subjects without FL. In FL of unspecified etiology, age was found to be an independent predictor of IMT and plaques, and steatosis had a protective effect in plaques only. Age was also recognized as the only independent predictor of carotid findings in NAFLD.

Although the prevalence of IMT among subjects with FL of unspecified etiology was increased, FL of unspecified etiology was not shown an independent predictor of IMT and was a negative predictor of plaques at logistic regression multinomial analysis; in contrast, GD and carotid plaques were shown to be associated. These findings can all be accounted for by the influence of age on the diseases in study. IMT and FL of unspecified etiology had the same distribution per age-classes, namely decreased in individuals aged > 40 years, whereas carotid atherosclerosis and GD increased in such over-40-year-old subjects (Figure 1). An increased prevalence of IMT among subjects with FL has also been reported in previous studies^[9,36]. However, while these authors reported the absolute value of IMT expressed in mm, we used a > 1-1.3 mm cut-off value, namely the threshold level predicting the occurrence of cardiovascular events^[37]. These methodological differences imply that we might have underestimated the true prevalence of IMT among subjects with FL of unspecified etiology to the advantage of predicting clinically relevant events. A recent study^[38] showed that after adjustment for confounders, NAFLD was significantly associated with an increased risk of cardiovascular diseases. However, additional adjustment for the metabolic syndrome appreciably attenuated this association without abolishing it. The finding that FL of unspecified etiology is an independent negative predictor of carotid plaques is probably linked to the inverse relationship linking age and body weight (Figure 3). Therefore, we emphasize that these data do not support a true “protective” effect of FL of unspecified etiology on the development of carotid plaques but probably represent the effect of selective mortality in those with FL and advanced AT, as suggested by the finding in this study that those with FL and IMT or plaques were 8 to 9 years younger than those with the same carotid findings without

FL.

Our data confirm the association between GD and carotid plaques^[19,20] but, in agreement with a previous study^[16], challenge that between GD and NAFLD. Both these findings are accounted for by the fact that carotid disease and GD both increase, whereas FL of unspecified etiology decreases, with age.

The importance of distribution per age-groups of subjects with various carotid findings, FL, NAFLD and GD also links the first (FL of unspecified etiology) and the second part (NAFLD) of our study. Individuals with NAFLD were significantly younger than those included in the first part of the study and thus they were less prone to the development of carotid plaques and GD. We highlight that in this subset of patients where all data were available, no other anthropometric, biological or histological parameters except for age, is an independent predictor of IMT and carotid plaques as found in the whole series. This finding fits with a previous study indicating that age is a powerful determinant of maximum IMT in both diabetic and healthy subjects^[39].

Some studies suggest that NAFLD patients have an increased incidence of carotid atherosclerosis^[9,40,41]. However, there is not enough hard data coming from natural history to support or rule out an association between NAFLD and cardiovascular disease. Indeed, some follow-up studies indicate that excess mortality in NAFLD is due to liver-related causes rather than to cardiovascular events^[12,13,35,42]. Enrollment criteria and referral pattern of patients might affect the vascular risk in NAFLD patients. NAFLD individuals included in our series were young and had a low prevalence of arterial hypertension and reduced HDL cholesterol that are strong determinants of AT^[43-45]. Furthermore, factors that are associated with the onset of carotid plaques might be different from those that cause clinical events of (complicated) AT.

Some specific features of our study need to be emphasized. First, the large series of subjects who were recruited. These individuals were distributed across all age-groups of adulthood, so providing an exhaustive cross-sectional picture of the relationship between the variables. Furthermore, our study enabled to evaluate those risk factors that add to the background dysmetabolic milieu linked to NAFLD *per se*. Indeed, our "control" group consisted of NAFLD patients with normal carotid findings rather than of subjects with a normal liver (i.e. without FL). Finally, we used the statistical procedure of multinomial logistic regression analysis which is particularly suitable in the analysis of unrelated multiple events, such as the varying outcomes of carotid ultrasonographic findings. We acknowledge, however, that ours is not a prospective study and that absence of anthropometric and biochemical data in the population with FL of unspecified etiology together with the limited number of NAFLD subjects may preclude exhaustive analysis of the etiological link between evaluated variables. Furthermore, the indications for prescribing ultrasound scanning of the liver or carotid depend on the clinical picture displayed by patients rather than by a standardized protocol. This may have reduced the number of subjects included in some age-groups (e.g. those aged 30 to 39 years) because these younger individuals seldom

present indications to undergo ultrasound scanning of carotid. This might somewhat bias our findings, that need to be duplicated by future studies.

In conclusion, our data show that in a large population comprising all ages of adulthood, age is an independent predictor of carotid IMT and plaques. FL of unspecified etiology is a negative predictor of carotid plaques, probably indicating selective death of those with FL or disappearance of liver fat with ageing. While we confirm an increased prevalence of IMT among patients with FL of unspecified etiology, and an association of GD with carotid atherosclerosis, we were unable to find any independent relationship between any conventional parameters, such as anthropometric, metabolic, hemodynamic, and histological liver features and carotid disease in NAFLD. In this specific subset of individuals, again, age is the only independent predictor of ultrasonographic carotid findings. Studies of natural history are needed to evaluate the real entity of cardiovascular risk in patients with FL of unspecified etiology and NAFLD as a function of age.

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CLINICAL RESEARCH

Hepatogenic differentiation of human mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells

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RESULTS: BMSC and ADSC exhibited a fibroblastic morphology that changed to a polygonal shape when cells differentiated. Expression of stem cell marker Thy1 decreased in differentiated ADSC and BMSC. However, the expression of the hepatic markers, albumin and CYPs increased to a similar extent in differentiated BMSC and ADSC. Hepatic gene activation could be attributed to increased liver-enriched transcription factors (C/EBP β and HNF4 α), as demonstrated by adenoviral expression vectors.

CONCLUSION: Mesenchymal stem cells can be induced to hepatogenic transdifferentiation *in vitro*. ADSCs have a similar hepatogenic differentiation potential to BMSC, but a longer culture period and higher proliferation capacity. Therefore, adipose tissue may be an ideal source of large amounts of autologous stem cells, and may become an alternative for hepatocyte regeneration, liver cell transplantation or preclinical drug testing.

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Key words: Mesenchymal stem cells; Bone marrow; Adipose tissue; Transdifferentiation; Hepatic lineage; Liver cell transplantation.

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Abstract

AIM: To investigate and compare the hepatogenic transdifferentiation of adipose tissue-derived stem cells (ADSC) and bone marrow-derived mesenchymal stem cells (BMSC) *in vitro*. Transdifferentiation of BMSC into hepatic cells *in vivo* has been described. Adipose tissue represents an accessible source of ADSC, with similar characteristics to BMSC.

METHODS: BMSCs were obtained from patients undergoing total hip arthroplasty and ADSC from human adipose tissue obtained from lipectomy. Cells were grown in medium containing 15% human serum. Cultures were serum deprived for 2 d before cultivating under similar pro-hepatogenic conditions to those of liver development using a 2-step protocol with sequential addition of growth factors, cytokines and hormones. Hepatic differentiation was RT-PCR-assessed and liver-marker genes were immunohistochemically analysed.

INTRODUCTION

Most liver diseases lead to hepatocyte dysfunction with the possibility of eventual organ failure. The replacement of diseased hepatocytes and the stimulation of endogenous or exogenous regeneration by stem cells are the main aims of liver-directed cell therapy. There is growing evidence to suggest that reservoirs of stem cells may reside in several types of adult tissue^[1,2]. These cells may retain the potential to transdifferentiate from one phenotype to another, presenting exciting possibilities for cellular therapies. One of

the important findings is that liver stem cells might be derived from bone marrow. Petersen *et al*^[3] first identified this phenomenon in a rat model of liver injury. Subsequently, many researchers have reported similar *in vivo* and *in vitro* findings^[4-8]. Bone marrow cells have been hypothesized as the third recruitment source in liver regeneration besides hepatocytes and endogenous liver stem cells^[9].

Human bone marrow, derived from the embryonic mesoderm, is a complex tissue formed by a population of hematopoietic stem cells (HSC), supported by a mesenchymal stroma. The bone marrow stroma is heterogeneous in composition and is a reservoir of several stem cell populations, such as mesenchymal stem cells (MSC), and multi-potent adult progenitor stem cells (MAPC)^[10]. It has been reported that MSC contributes to the regeneration of a variety of mesenchymal tissues. Furthermore, it retains the ability to differentiate into cells of the mesoderm lineage, such as osteoblasts, chondrocytes, adipocytes, myoblasts and cardiomyocytes, and into various types of tissue cells derived from other embryonic layers, including neural and liver cells^[11-16]. The transdifferentiation of BMSC into hepatic cells *in vivo* was described in rats^[3,17], mice^[7] and humans^[8,18]. This has brought new hope to cell therapy using autologous bone marrow cells as these present few ethical problems and could be applied to severe liver disease. MSC can easily be obtained following a simple bone marrow aspiration procedure, and may be subsequently cultured and expanded *in vitro* without losing their stem cell potential, making them an attractive target for cell therapy. In addition to long-term self-renewal capability, MSC possess versatile differentiation potential ranging from mesenchyme-related multipotency to neuroectodermal and endodermal competency. However, traditional bone marrow procurement procedures may be distressful for the patient and may yield low numbers of MSCs upon processing, which has recently led to investigate alternative MSC sources outside the bone marrow microenvironment.

Although bone marrow was the first source reported to contain MSC, it has been reported that MSC can also be isolated from human umbilical cord blood, synovium, placenta, periosteum, skeletal muscle, and adipose tissue^[1,19-24]. Adult adipose tissue, like bone marrow, is derived from the embryonic mesenchyme, and a putative stem cell population within the adipose stromal compartment has been identified. Adipose tissue represents a rich source of mesenchymal stem cells, and provides an abundant and accessible source of adult stem cells with minimal patient discomfort. These cells have been termed adipose tissue-derived stem cells (ADSC). The characterization of these ADSC has defined a population similar to bone marrow-derived and skeletal muscle-derived stem cells. This cell population can be isolated from human lipoaspirates, and can be differentiated toward the osteogenic, adipogenic, neurogenic, myogenic and chondrogenic lineages^[25-29] like bone marrow BMSC. In fact, some works have shown that human ADSC have similar characteristics to BMSC *in vitro* and *in vivo*^[22,23,30,31]. In addition, ADSC compared with MSC from other sources possessed the longest culture period and the highest proliferation capacity^[23]. Thus, adipose tissue may

be an ideal source of large amounts of autologous stem cells attainable by a less invasive method than BMSC.

The success seen in differentiating ADSC into various mesenchymal lineages generates great interest in the use of ADSC for hepatic differentiation. To our knowledge, however, only a very recent report has shown the hepatogenic differentiation potential of ADSC^[32] by using a differentiation protocol showing significant differences to the one used in this study. In addition, a comparative study of the hepatogenic differentiation potential *in vitro* of both types of MSC has not yet been performed.

Based on these previous findings, the aim of this study was to investigate and compare the hepatic differentiation of human MSC from bone marrow (BMSC) and adipose tissue (ADSC) obtained from healthy donors. To this end, these cells were isolated and cultured under similar pro-hepatogenic conditions to those of liver development to define the different capacities of hepatic differentiation of the two subsets *in vitro*. Briefly, BMSC and ADSC cultures were serum deprived for 2 d and pre-cultured with EGF and bFGF (conditioning step). Then a 2-step differentiation protocol followed with a sequential addition of growth factors, cytokines and hormones (step-1 differentiation: HGF and bFGF for 7 d and step-2 differentiation: OMS, dexamethasone, and ITS + up to d 21). Moreover, the response to inductive extracellular signals and the role of key liver-enriched transcription factors (LEFTs) in the differentiation process *in vitro* have been revealed.

MATERIALS AND METHODS

Materials and reagents

Medium Dulbecco's Modified Eagle's medium (DMEM-low glucose), Ham's F-12 and Leibovitz L-15 were purchased from Gibco (Paisley, UK), gentamicine from Normon (Madrid, Spain), phosphate-buffered saline (PBS) and dexamethasone from Merck Pharma (Mollet del Vallés, Spain) and trypsin-EDTA and newborn calf serum were obtained from Biochrom AG (Berlin, Germany), ITS + premix from BD Biosciences (Madrid, Spain). Basic fibroblast growth factor (bFGF) was purchased from Invitrogen (Barcelona, Spain), epidermal growth factor (EGF) and nicotinamide were from Sigma-Aldrich (Madrid, Spain). Oncostatin M (OMS) was purchased from PeproTech EC (London, UK). Hepatocyte growth factor (HGF) was a kind gift from Dr. T. Nakamura (Tokohu University, Sendai, Japan). Trizol and DNase I Amplification Grade were purchased from Invitrogen, Life Technologies (Barcelona, Spain), 3,3'-diaminobenzidine from Sigma-Aldrich (Madrid, Spain) and toluidine blue from BDH chemicals (Poole, UK). Monoclonal antibodies against human antigens CD13-PE, CD34-APC, CD45-FITC, CD90-APC, were purchased from Becton Dickinson (Mountain View, CA); CD105-PE from Serotec (Oxford, UK) and 7-Amino-actinomycin D (7-AAD) from Sigma-Aldrich (Madrid, Spain). Mouse monoclonal anti-human alphafetoprotein was purchased from Santa Cruz Biotechnology (Madrid, Spain) and polyclonal rabbit anti-human albumin, alphafetoprotein, polyclonal goat anti-mouse HRP and polyclonal goat anti-rabbit HRP were from DakoCytomation (Barcelona, Spain).

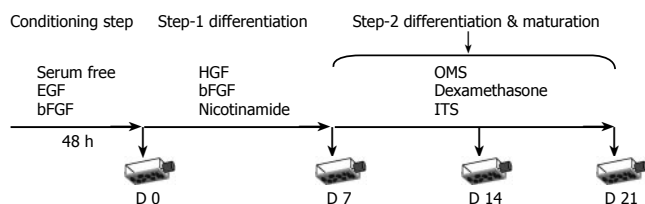


Figure 1 Hepatic differentiation protocol by sequential addition of exogenous factors according to embryogenesis. Passage 2 cultures at 85% of confluency were used for differentiation assays. Cells were pre-cultured in serum-free medium supplemented with EGF and bFGF for 2 d (Conditioning step). Then cells were cultured in medium supplemented with HGF, bFGF and nicotinamide, for 7 d (Step-1 differentiation). Finally cells were cultured in maturation medium supplemented with OMS, dexamethasone, and ITS + premix (insulin, transferrin, selenious acid, BSA and linoleic acid) up to 21 d (Step-2 differentiation; maturation). Media were changed twice a week and hepatic differentiation was assessed at different time points.

Cell cultures

Cancellous bone was obtained from the femoral heads of five patients (aged between 60 and 69 yr) undergoing total hip arthroplasty. Human adipose tissue was obtained by suction-assisted lipectomy (i.e. liposuction) as discarded tissue from surgical interventions from six patients (aged between 38 and 49 years). These protocols were approved by the institutional review board. Tissues were obtained in conformity with the rules of the Hospital's Ethics Committee and after obtaining an informed consent from patients.

Both bone and fat were dissected into small pieces. Trabecular bone fragments were digested with collagenase II (3 mg/mL) in Hank's balanced salt solution (HBSS) at 37°C for two hours with intermittent shaking. Adipose tissue fragments were digested with collagenase I (1 mg/mL) in HBSS at 37°C for 60 min with intermittent shaking. Thereafter, the resulting suspensions (bone and fat) were filtered using two layers of cotton gauze to remove debris and then centrifuged at 400 *g* for 10 min. Supernatants were discarded and pellets were resuspended in 160 mmol/L NH₄Cl at room temperature for 10 min to lyse the remaining red blood cells. Cells were collected by centrifugation, as detailed above, resuspended in culture medium (DMEM-low glucose supplemented with 15% AB human serum stock (pooled from 10 donors) from our regional transfusion centre and 50 µg/mL of gentamicine). These primary cells were plated in tissue culture flasks in a humidified atmosphere at 37°C with 50 mL/L CO₂ for 48 h. Thereafter, the cultures were washed to remove non-adherent cells. Media were changed twice a week, maintaining cells at subconfluent levels. The cells were subcultured with 0.4% trypsin/0.2% EDTA solution, seeded at a density of 5-10 × 10³ cells/cm² and cultured in the same conditions used for the primary culture (passage 1). Five to seven days later, subconfluent monolayers were subcultured (passage 2) and cells were seeded at the same density as for passage 1 to be used for differentiation assays.

Cell lines

HepG2 cells were plated in Ham's F-12/Leibovitz L-15 (1:1 v/v) supplemented with 7% newborn calf serum, 50 U penicillin/mL and 50 µg streptomycin/mL. Cells were subcultured with 0.25% trypsin/0.02% EDTA at 37°C.

Table 1 Sequence of primers used for RT-PCR and length of fragments

Primer	Sequences	Product
ALB	Upper 5' TGAGAAAACGCCAGTAAGTGAC 3' Lower 5' TGCAGAAATCATCCATAACAGC 3'	265 bp
AFP	Upper 5' GCTTGGTGGTGGATGAAACA 3' Lower 5' TCCTCTGTTATTTTGGCTTTTG 3'	157 bp
Thy1	Upper 5' CACACATACCGCTCCCGAACC 3' Lower 5' GCTGATGCCCTCACACTTGACC 3'	189 bp
KRT 18	Upper 5' CCCGTCACGCCCTACAGAT 3' Lower 5' ACCACTTTGCCATCCACTATCC 3'	271 bp
KRT 19	Upper 5' TCCAGATGAGCAGGTCCGAGGTTA 3' Lower 5' GCTGCGGTAGGTGGCAATCTCC 3'	281 bp
CYP3A4	Upper 5' CCTTACAT TACACACCTTTGGAAGT 3' Lower 5' AGCTCAATGCATGTACAGAATCCCGGTTA 3'	382 bp
CYP2E1	Upper 5' ACAGAGACCACCAGCACAAC 3' Lower 5' ATGAGCGGGGAATGACACAGA 3'	580 bp
C/EBPβ	Upper 5' CTCGCAGGTCAAGAGCAAG 3' Lower 5' CTAGCAGTGGCCGAGGCGAGC 3'	271bp
HNF4α	Upper 5' GCCTACCTCAAAGCCATCAT 3' Lower 5' GACCCTCCCAGCAGCATCTC 3'	275 bp
hPBGD	Upper 5' CGGAAGAAAACAGCCCAAAGA 3' Lower 5' TGAAGCCAGGAGGAAGCACAGT 3'	294 bp

Hepatic differentiation protocol

Passage 2 BMSC and ADSC cultures at 85% confluency were used for differentiation assays. Cells were serum deprived for 2 d and pre-cultured in DMEM supplemented with 20 ng/mL EGF and 10 ng/mL bFGF (conditioning step) to stop cell proliferation, prior to induction of differentiation toward a hepatic phenotype. Then a 2-step differentiation protocol was performed, followed by a sequential addition of growth factors, cytokines and hormones (Figure 1). Step-1 differentiation medium, consisting of DMEM supplemented with 20 ng/mL HGF, 10 ng/mL bFGF and nicotinamide 4.9 mmol/L, for 7 d, followed by step-2 differentiation medium, consisting of DMEM supplemented with 20 ng/mL OMS, 1 µmol/L dexamethasone, and 10 µL/mL ITS + premix (final concentration: 100 µmol/L insulin, 6.25 µg/mL transferrin, 3.6 µmol/L selenious acid, 1.25 mg/mL BSA and 190 µmol/L linoleic acid) to achieve cell maturation up to D21. Media were changed twice weekly and hepatic differentiation was assessed at different time points by RT-PCR for liver-associated genes, as listed in Table 1. The protocol was applied to BMSC and ADSC from five and six different donors, respectively, and identical results were obtained.

Flow cytometry analysis

Flow cytometric analysis was performed with a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA) after labelling 1 × 10⁶ cells with respective directly conjugated antibodies, using isotype-matched controls. 7-Aminoactinomycin D was used in order to exclude non-viable cells from the analysis, and nonspecific staining was performed as previously described^[33]. This analysis was carried out after the initial sample was obtained and later in the following culture stage.

Immunohistochemistry

MSC in monolayer were washed twice with phosphate buffered saline (PBS), fixed with ethanol 70% for 10 min, and with ethanol 100% for 10 min thereafter. Plates were washed three times with PBS. Then, plates were incubated for 30 min at room temperature with a solution of methanol with 1.7% H₂O₂, and washed again with PBS. Plates were then incubated for 30 min at 37°C with sheep serum diluted 1/10 in PBS, and washed with PBS tween (0.05%) four times. Subsequently, the plates were incubated for 2 h at 37°C with the primary antibody diluted in PBS tween 0.05%: anti-human alphafetoprotein (1/50) and anti-human albumin (1/2000). After incubation, plates were washed with PBS tween (0.05%) four times and incubated for 1 hour at 37°C with peroxidase labelled goat anti-mouse IgG diluted 1/100 in PBS tween 0.05% for alphafetoprotein, and with peroxidase labelled goat anti-rabbit IgG diluted 1/1000 in PBS tween 0.05% for albumin. After repeated washes, plates were incubated with the substrate (3,3'-diaminobenzidine diluted in PBS with 0.1% H₂O₂). H₂O was used to stop the reaction, and toluidine blue was employed as contrast staining. Finally, plates were examined under the microscope.

RNA extraction and real-time quantitative PCR

Total RNA was extracted from undifferentiated (used as control), differentiating and differentiated cells using Trizol reagent, following the manufacturer's recommendations; contaminating genomic DNA was removed by incubation with DNase I Amplification Grade. The amount of purified RNA was estimated by ribogreen fluorescence (Molecular Probes), and its purity was assessed by the absorbance ratio 260/280 nm. RNA integrity was examined by agarose gel electrophoresis. RNA (1 µg) was reverse transcribed as described^[34,35]. Diluted cDNA (3 µL) was amplified with a rapid thermal cycler (LightCycler Instrument, Roche Diagnostics) in 15 µL of LightCycler DNA Master SYBR Green I (Roche Molecular Biochemicals), 5 mmol/L MgCl₂ and 0.3 µmol/L of each oligonucleotide. Optimal MgCl₂ concentration was empirically determined for each set of primers. Gene-specific primers were designed using OLIGO software. The sequence of both the forward and reverse primers and the expected sizes of the PCR-amplified DNA are listed in Table 1. In parallel, we analyzed the mRNA concentration of the human housekeeping porphobilinogen deaminase (PBGD, hydroxymethylbilane synthase) as an internal control for normalization. A no-template negative control (H₂O) was also run for every cDNA-specific primer set. Each sample cDNA was measured in duplicate. PCR amplicons were confirmed to be specific by size and melting curve analysis. After denaturing for 30 s at 95°C, amplification was performed with 40 cycles of 1 s at 94°C, 5 s at 62°C and 20 s at 72°C. The real-time monitoring of the PCR reaction, the precise quantification of the products in the exponential phase of the amplification and the melting curve analysis were performed with the LightCycler quantification software, as recommended by the manufacturer.

Transduction with adenoviral vectors encoding C/EBPα and β

CCAAT/enhancer-binding proteins (C/EBP) are a family of liver-enriched transcription factors, which play an important role in regulating the transcription of multiple hepatic genes, including CYPs. Recombinant adenoviruses encoding the C/EBPα and C/EBPβ full-length proteins were prepared as described elsewhere^[36,37]. Briefly, recombinant shuttle pAC/CMVpLpA plasmids containing full-length C/EBPα and C/EBPβ cDNAs were cotransfected into 293 cells with pJM17 (AdE1A-transformed human embryonic kidney cells) by calcium phosphate/DNA coprecipitation. The expression cassette of pAC/CMVpLpA is located between the sequences representing 0-1.3 mu and 9.2-16 mu of adenovirus type 5, whereas pJM17 encodes a full length adenovirus-5 genome (dl309) interrupted by the insertion of the bacterial plasmid pBRX at position 3.7 mu, thereby exceeding the packaging limit for the adenovirus. A homologous recombination between adenovirus sequences in the shuttle vector and in the pJM17 plasmid results in the substitution of the pBRX sequences in pJM17 by the chimeric gene. This generates a genome of a packageable size in which most of the adenovirus early region 1 is lacking, thus rendering the recombinant virus replication defective. The resulting viruses (called Ad-C/EBPα, Ad-C/EBPβ) were plaque-purified, expanded into a high-concentration stock and titrated by plaque assay, as previously described^[38]. ADSCs and BMSCs were infected with recombinant adenoviruses for 120 min at a MOI (multiplicity of infection) ranging from 3 to 15 PFUs (plaque forming units) per cell (MOI). Thereafter, cells were washed with PBS and fresh medium was added. Forty-eight hours post-transduction cells were analyzed or harvested for analysis and frozen in liquid N₂. MSCs were also transduced with a control adenoviral vector expressing GFP to confirm a highly efficient transduction of adenovirus exposed human cells and to demonstrate that adenoviral transduction *per se* does not cause unspecific alterations in differentiation marker genes.

Statistical analysis

Data were expressed as mean ± SE. Results were analysed by the Student's *t* test. *P* < 0.05 was considered statistically significant.

RESULTS

Differentiation of human BMSC and ADSC to hepatic phenotype

Analysis by flow cytometry of cell surface markers of BMSC and ADSC after isolation was performed.

Homogeneity and reproducibility of the isolation procedure of BMSC and ADSC were demonstrated by flow cytometry. Two analytical regions were established combining R1 region, which selects viable cells, and R2 in forward and side characteristics of both MSC types (Figures 2A, B and 3A, B). The surface antigen markers of human mesenchymal stem cells CD13, CD45, CD90 and CD105 expression on BMSC and ADSC were analysed after isolation

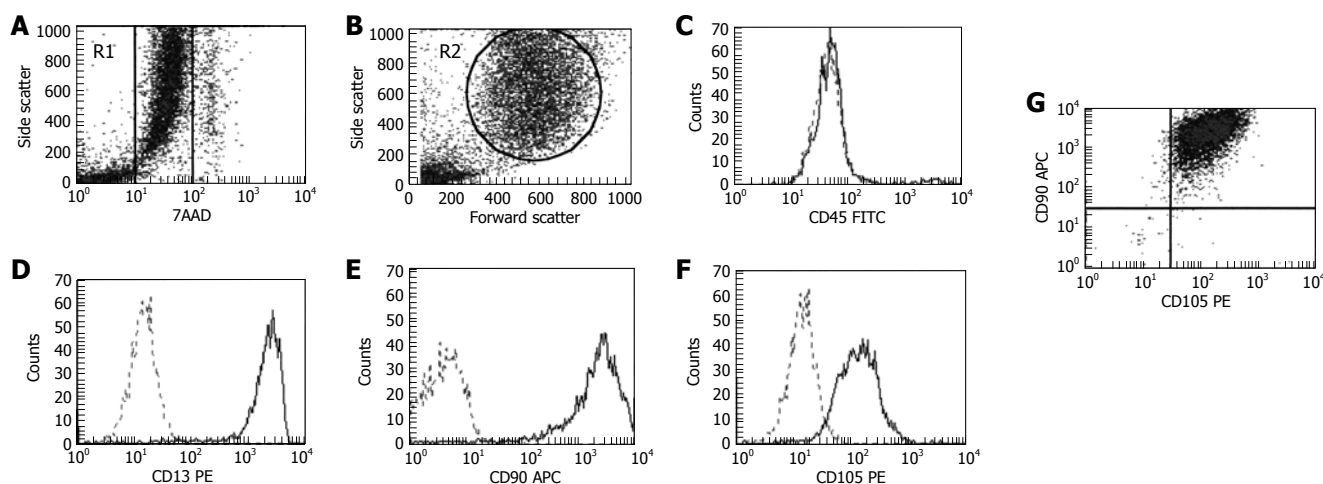


Figure 2 Flow cytometry analysis of surface protein markers of BMSC. Two analytical regions were established combining R1 region (A), which selects viable cells, and R2 (B) in forward and side characteristics of BMSC. Surface antigen markers were positive vis-à-vis isotypic controls for CD13 (D), CD90 (E) and CD105 (F) and negative for CD45 (C). Co-expression of CD90-CD105 is shown in dot-plots (G). 7ADD: 7-Aminoactinomycin D.

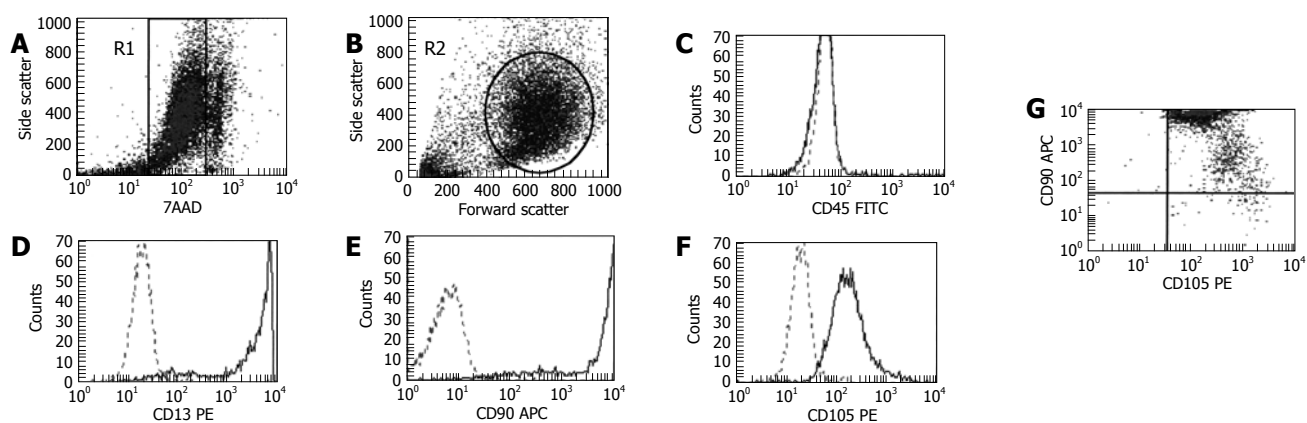


Figure 3 Flow cytometry analysis of surface protein markers of ADSC. Two analytical regions were established combining R1 region (A), which selects viable cells, and R2 (B) in forward and side characteristics of ADSC. Surface antigen markers were positive vis-à-vis isotypic controls for CD13 (D), CD90 (E) and CD105 (F) and negative for CD45 (C). Co-expression of CD90-CD105 is shown in dot-plots (G). 7ADD: 7-Aminoactinomycin D.

and at different steps of the culture. Representative histograms for BMSC and ADSC are shown in Figures 2 and 3 respectively. Both types of undifferentiated MSC were positive for the mesenchymal markers CD13 (Figures 2D and 3D), CD90 (Thy1) (Figures 2E and 3E) and CD105 (Figures 2F and 3F), but did not express the hematopoietic marker CD45 (Figures 2C and 3C). As shown in Figures 2G and 3G, the coexpression of both antigens CD90 (Thy1) and CD105 is found in ADSC and BMSC. ADSC showed an identical pattern of surface protein expression as BMSC, and no significant variation of surface marker expression was observed among cells from different donors. Table 2 shows that the percentage of positive cells after the primary culture of BMSC and ADSC is similar. Data for the mean fluorescence intensity for the markers CD90 and CD105 show differences between individual donors with a mean fluorescence greater for both markers in ADSC than BMSC. Nevertheless these last data may take into account the variations in the photomultiplier settings of the cytometer for the timing of the different cultures.

Morphologic changes in cultured ADSC and BMSC

We analysed the morphological changes of BMSC and ADSC at the various differentiation protocol stages in order to evaluate the effect of growth factors, hormones and cytokines. Both MSC obtained from human adipose tissue and human bone marrow expanded easily *in vitro*. However, ADSC showed a higher proliferation rate and longer survival in culture than BMSC. When cells were pre-cultured for 2 d in serum-free medium supplemented with EGF and bFGF, cell proliferation stopped. Cells before differentiation (D0) exhibited a fibroblast-like morphology (Figure 4A and B). Cell morphology of both BMSC (Figure 4C) and ADSC (Figure 4D) did not change significantly during differentiation step-1, when cultures were treated with HGF, although the fibroblastic morphology was lost and cells developed a broadened flattened shape. However, a polygonal shape developed during differentiation step-2 when cells were exposed to media containing OMS and hormones (Figure 4E and F). The protocol used includes the sequential addition of exogenous factors that have

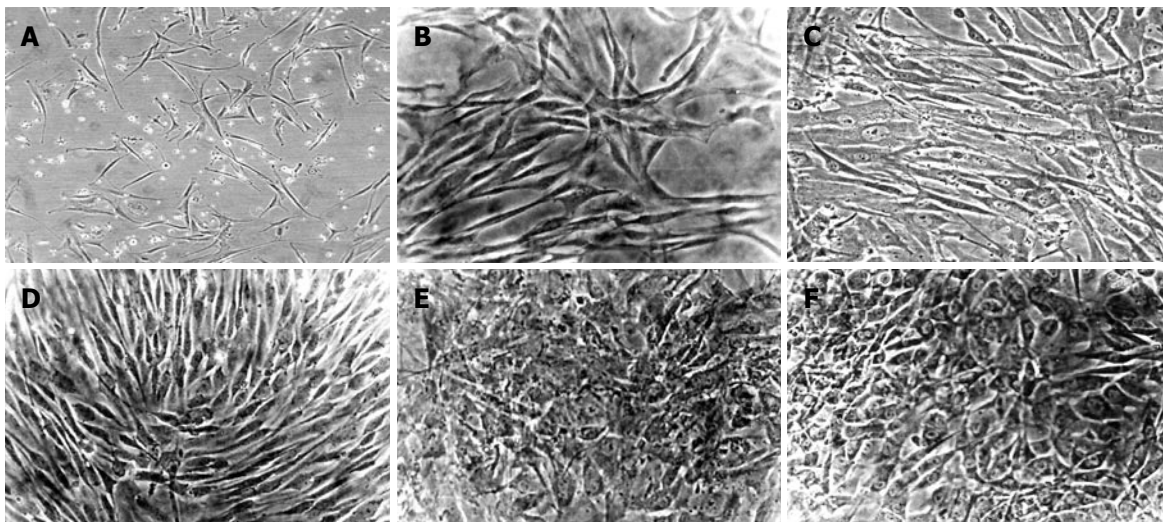


Figure 4 Morphology of human mesenchymal stem cells from bone marrow and adipose tissue during the differentiation protocol. Cells were induced to differentiate by using a sequential addition of growth factors, cytokines and hormones. Morphology of passage 2 BMSC (A) and ADSC (B) cells. No significant morphological changes were observed in BMSC (C) nor in ADSC (D) cells during the step-1 differentiation. However, both BMSC (E) and ADSC (F) cells significantly changed the morphology, and developed a polygonal shape during step-2 differentiation (magnification 20 x for all pictures).

Table 2 Expression of CD90 and CD105 in ADSC and BMSC after the initial culture

Donor	ADSC				BMSC			
	CD90 ⁺		CD105 ⁺		CD90 ⁺		CD105 ⁺	
	Cell population %	Mean channel fluorescence	Cell population %	Mean channel fluorescence	Cell population %	Mean channel fluorescence	Cell population %	Mean channel fluorescence
1	99.88	8623.91	93.92	211.55	98.83	8347.11	94.85	338.36
2	99.83	5467.01	99.83	1118.22	87.69	1980.05	92.20	175.20
3	99.82	6157.88	99.08	1089.24	99.80	8538.72	96.27	129.80
4	99.89	6830.06	97.79	758.79	98.17	8700.00	89.48	622.80
5	99.78	7495.60	99.70	2025.79	98.91	8562.77	95.53	602.95
6	99.81	8052.66	99.54	1510.95	76.74	1088.24	98.70	139.73
7	98.83	8878.06	99.35	1298.27	76.42	234.83	89.20	648.32
8	91.14	5123.70	73.52	203.77	98.98	6274.51	94.90	549.53
Mean	98.62	7078.61	95.34	1027.07	91.94	5465.78	93.89	400.84
SD	3.04	1418.10	9.03	624.75	10.26	3724.95	3.33	229.88

been reported to be implicated in liver development and proved to be effective to induce the hepatic differentiation of human MSC from human bone marrow and umbilical cord blood^[39].

RT-PCR analysis of hepatic gene expression of BMSC and ADSC differentiated cells

To determine whether differentiated cells show the characteristic expression of hepatic phenotype markers, total RNA from BMSC and ADSC was isolated at D7, 14 and 21 of the differentiation protocol and the mRNA levels of several hepatic genes were examined by RT-PCR. Undifferentiated cells were used as controls (D0 of the differentiation protocol). The CK-18 and CK-19 expression did not significantly change by induction of differentiation (data not shown). Expression of HNF4 α , C/EBP β (liver-enriched transcription factors that play important roles in regulating the expression of hepatic genes) and albumin were significantly increased with the

differentiation time in both cell types, BMSC and ADSC. However, the expression of hepatocyte specific markers in ADSC occurs predominantly in the first differentiation step, whereas the second differentiation step is required in BMSC. It is unclear whether this fact could imply functional differences between both cell populations, and further research is needed to clarify this point. The time-course induction of C/EBP β correlated well with albumin upregulation (Figure 5A-C), while HNF4 α induction showed a different profile (Figure 5D). The increase in key liver-enriched transcription factors suggests that our differentiation protocol was effective in driving MSC toward a hepatic phenotype.

Then, we went on to compare the levels of the Thy1 (CD90) and alphafetoprotein (AFP) expressions in ADSC and BMSC with that of adult human liver tissue from a liver bank (pooled liver from 10 donors). To this aim, we performed quantitative RT-PCR relative to housekeeping hPBGD at the initial and final times of the differentiation

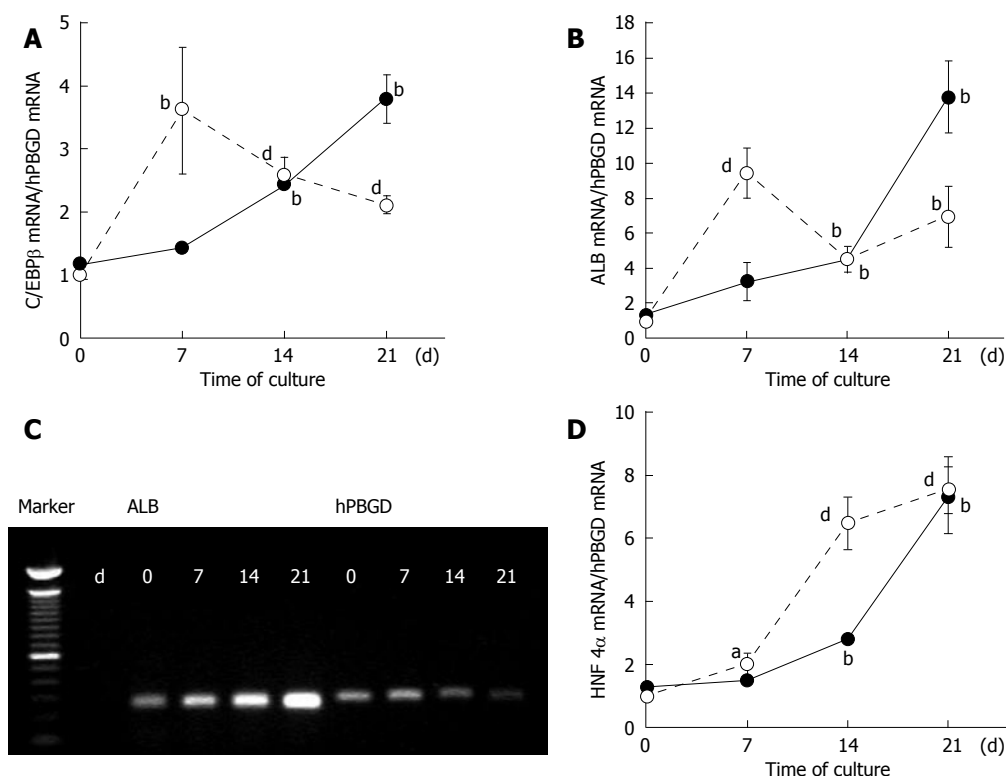


Figure 5 Real-time PCR analysis of the expression of mRNA for liver markers determined at established times in the transdifferentiation protocol of BMSC and ADSC. Expression of C/EBPβ (A) (liver-enriched transcription factor, that plays important roles in regulating the expression of hepatic genes), albumin (B and C) and HNF4α (D), were analysed in BMSC (bold symbols) and ADSC (clear symbols) at different time points of the differentiation protocol. Data are shown as the fold increase in the mRNA level compared to the undifferentiated cells (d 0 of the differentiation protocol), and were normalized by hPBGD. The agarose gel shows the ALB expression of BMSC (C). HNF4α: hepatocyte nuclear factor 4 alpha; C/EBPβ: CCAAT / enhancer binding protein beta; ALB: albumin. Data are the mean \pm SE of 5 and 6 different cultures of BMSC and ADSC respectively. ^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$.

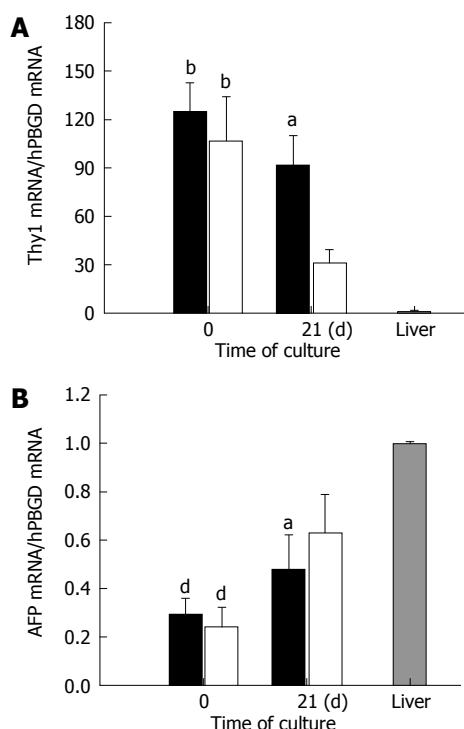


Figure 6 Real-time PCR analysis of the expressions of mRNA for Thy1 and alphafetoprotein in BMSC and ADSC. The levels of Thy1 (CD90) (A) and the alphafetoprotein (B) expression in differentiated BMSC (black bars) and ADSC (clear bars) were quantified by RT-PCR at the initial and final times of the differentiation protocol, and compared with that of human pooled liver tissue from a liver bank. Data are shown as a fold increase in the mRNA level compared to the liver tissue and normalized by hPBGD. AFP, alphafetoprotein. Data are the mean \pm SE of 4 different cultures of BMSC and ADSC. ^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$.

protocol. The relative mRNA levels of the mesenchymal marker Thy1 in undifferentiated ADSC and BMSC was significantly higher ($P < 0.01$), than in human liver, as expected (Figure 6A). However, after 21 d of the

differentiation protocol ADSC significantly reduced the Thy1 expression level, while a more modest decrease was observed in BMSC ($P < 0.05$). AFP is considered a characteristic fetal hepatic marker. AFP expression levels in fetal mouse liver steadily increases 1000-fold from day 9.5 to 15.5 of gestation and drops dramatically in adult liver. At low levels however, AFP is still reproducibly detected in multiple adult liver samples by real-time RT-PCR. Regarding AFP, a similar level of expression was observed in undifferentiated ADSC and BMSC, where it was significantly lower than that in liver ($P < 0.001$). However, differences with liver tissue were reduced in ADSC (NS) and BMSC ($P < 0.05$) after the step-2 protocol (Figure 6B). In the less differentiated human hepatoma HepG2 cells, AFP expression was much higher than in differentiated MSCs or liver tissue (data not shown).

We also determined the expression of CYPs (CYP2E1 and CYP3A4) as well as the differentiated markers of adult hepatic phenotype in ADSC and BMSC. We compared expression levels by RT-PCR at the initial and final times of the differentiation procedure (Figure 7). Levels of CYP2E1 in the undifferentiated MSC were not significantly different from that of the human hepatoma HepG2, a cell line showing characteristics of fetal hepatocytes. However, a significant increase of CYP2E1 expression was observed at the final differentiation time in both ADSC ($P < 0.01$) and BMSC ($P < 0.001$) (Figure 7A). Levels of CYP3A4 at the initial time were significantly lower in MSC when compared with HepG2 ($P < 0.001$), but these levels increased in the differentiated cells: BMSC showed a similar mRNA level while ADSC reached a higher expression level than the HepG2 cells ($P < 0.001$) (Figure 7B and C). The expression level of CYP2E1 and CYP3A4 in differentiated MSCs was still lower than in human hepatocytes (data not shown), which suggests that

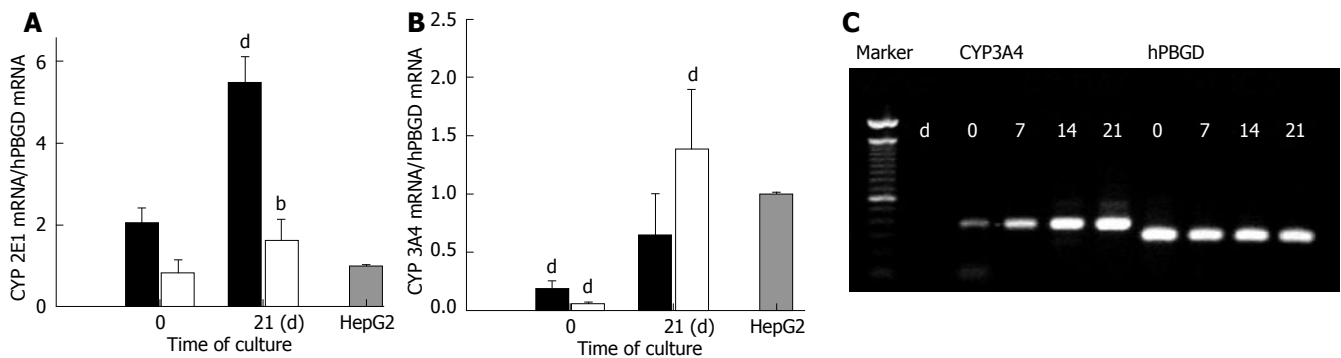


Figure 7 Real-time PCR analysis of the expression of mRNA for CYP2E1 and CYP3A4 in BMSC and ADSC. The expression of two major drug metabolising enzymes, CYP2E1 (A) and CYP3A4 (B), in BMSC (black bars) and ADSC (clear bars) were quantified by RT-PCR at the initial and final times of the differentiation protocol, and compared with that of the human hepatoma cells HepG2. The agarose gel shows the CYP3A4 expression of ADSC (C). Data are shown as a fold increase in the mRNA level compared to HepG2, and were normalized by hPBGD. Data are the mean \pm SE of 3 different cultures of BMSC and ADSC. ^a $P < 0.01$; ^b $P < 0.001$.

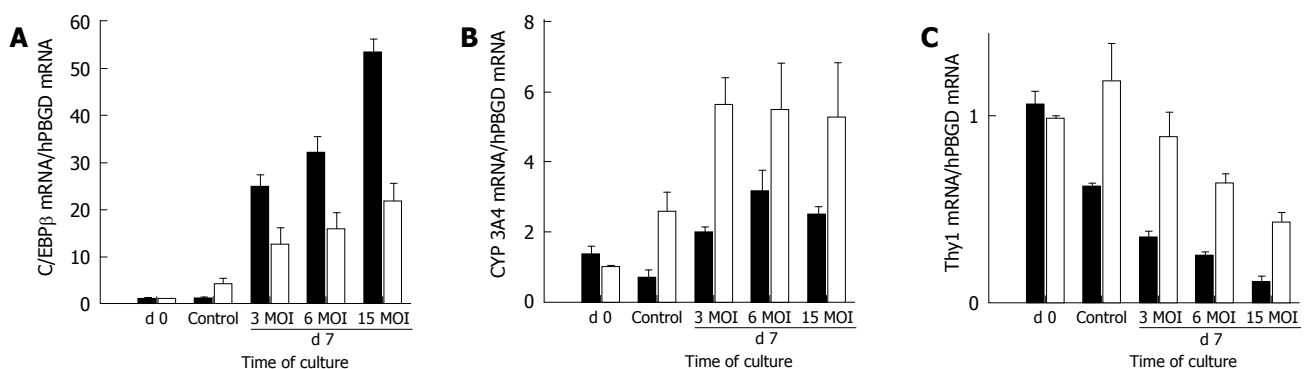


Figure 8 Evaluation of the role of C/EBP transcription factors in the differentiation of BMSC and ADSC cells. Adenoviral transduction caused a dose-dependent increase in the level of C/EBPβ mRNA, as assessed by real-time PCR analysis (A). Concomitantly, an up-regulation of CYP3A4 was observed which was more significant in ADSCs (B), and was paralleled by a significant down-regulation of the mesenchymal cells marker Thy1 in both BMSC and ADSC (C). Controls were not transduced. Data are shown as a fold increase in the mRNA level compared to the control and normalized by hPBGD. Data are the mean \pm SE of 3 different cultures of BMSC and ADSC.

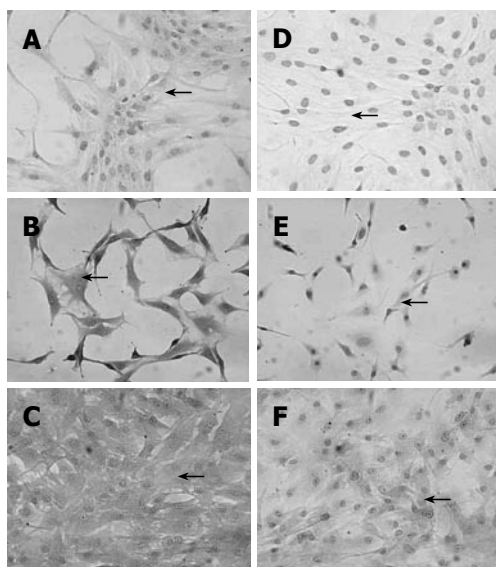


Figure 9 Immunohistochemical analysis of albumin and alpha-fetoprotein in ADSC cells. Homogeneous expression of albumin (A-C) and AFP (D-F) in ADSC cells was further confirmed by immunocytochemistry. Staining for both albumin and AFP was negative in undifferentiated cells (A,D). ADSC cells homogeneously increase their protein expression levels in response to the differentiation protocol. Positive staining was shown after 14 d (B, E) and 21 d (C, F) of the 2-step differentiation protocol. AFP; alpha-fetoprotein.

additional stimuli may be needed to reach adult physiologic expression levels of CYP genes. By taking these results together it is difficult to clarify whether ADSC and BMSC are in fact equally potent hepatic progenitor cells, and further functional studies are required.

Role of C/EBP transcription factors in mesenchymal to hepatic transition

We observed a significant increase in the expression of key liver transcription factors during the transdifferentiation of MSC into the hepatic phenotype. To investigate the relevance of this finding, we transduced undifferentiated cells with adenoviral vectors encoding full-length C/EBP proteins and analyzed their effects by driving MSC towards the hepatic phenotype. The most important results were found with C/EBPβ, while C/EBPα appeared to play a minor role.

Cells were transduced after seven d of culture with increasing doses of adenoviruses, ranging from 3-15 MOI. Infection of MSC with a control adenovirus expressing the GFP demonstrated an 85%-95% transduction efficiency. Moreover, transduction of MSC with Ad-GFP or Ad-PAC (insertless adenoviral vector) up to 15 MOI neither modified the morphology nor the expression of

differentiation marker genes, demonstrating that all the effects observed in Ad-C/EBP transduced cells can be attributed to the increased expression of the adenoviral transgene (data not shown). As expected, adenoviral transduction caused a dose-dependent increase in the level of C/EBP β mRNA (Figure 8A). Concomitantly, we observed an up-regulation of CYP3A4, which was more significant in ADSCs (Figure 8B), and expression levels above those found in well-differentiated hepatoma cells were reached. In parallel, we observed a significant down-regulation of the mesenchymal marker Thy1 (Figure 8C), suggesting that the C/EBP β up-regulation is an important event in triggering the mesenchymal to hepatogenic transition.

Immunohistochemistry

To further confirm the homogeneous expression of albumin and AFP in our cell populations, we examined differentiated ADSC by immunocytochemistry. This analysis showed that undifferentiated cells stain negative for albumin and AFP (Figure 9A and D). Positive staining was detected at the end of step-1 differentiation when cells had been exposed to HGF (Figure 9B and E), and a more intense positive staining was found at d 21 at the end of step-2 differentiation, when cells were cultured with OSM and hormones (Figure 9C and F). Our results demonstrate that the levels of hepatic protein markers increased homogeneously in most of the ADSC cells in response to the differentiation protocol, correlating well with mRNA expression analysis.

DISCUSSION

Over recent years, stem cells have generated great interest given their potential therapeutic use. Bone marrow has been the best characterized tissue, and has been considered as the major source of multipotent adult MSC since it is able to differentiate into mesodermal and ectodermal lineages^[11-16]. Several studies have indeed shown the potential of BMSC to differentiate into hepatocyte-like cells^[3,7,8,17,18]. However, the presence of uncommitted MSC has been found in many other tissues, such as adipose tissue, thus representing an alternative source of multipotent stromal cells^[1,19-22]. In fact, human ADSC have similar characteristics to BMSC^[22,23,30,31]. In addition, Zuk *et al*^[22] reported that ADSC are capable of differentiating *in vitro* into several cell lineages. Moreover, it has been recently shown for the first time that human ADSC can differentiate towards hepatic lineage *in vitro* under appropriate culture conditions^[22]. Therefore, adipose tissue may be readily accessible and a good candidate as a source of stem cells with a therapeutic potential for hepatic cell therapy and tissue engineering. Although this is a controversial area, some studies clearly show that transplanted bone-marrow-derived hepatocytes can colonise a wide variety of tissues in the body of a host due to cell fusion^[40,41], while others do not report cell fusion^[42].

Although the stem cell differentiation mechanism remains unclear to date, transdifferentiation might either be induced by stimulating with suitable media/substrates/factors, or by genetic reprogramming *in vitro*^[43-45]. Liver

development is known to proceed *via* several distinct steps in which growth factors and cytokines are involved. Among the factors implied in the embryonic liver development, fibroblast growth factors (FGFs), produced by cardiac mesodermal cells, are involved at an initial stage of endodermal patterning to induce hepatic fate^[46,47]; OSM, a member of the interleukin-6 cytokine family produced by hematopoietic cells, is required from the mid-fetal to the neonatal stages^[48] and apparently coordinates liver development and hematopoiesis in the fetus^[49]; finally, several extracellular signals including EGF, HGF, OSM, FGFs, glucocorticoids and insulin are involved in the late maturation stage leading to an increase in liver-specific gene expressions, and their effects on differentiation vary as a function of gestation age^[50]. Corticosteroids, HGF and EGF play important roles in hepatic biology^[51]. HGF is a more potent proliferating factor for human hepatocytes in culture than EGF^[52,53], and plays an important role in liver development and regeneration in humans^[54,55]. The differentiation of BMSCs into hepatocyte-like phenotypes *in vitro* by induction with HGF has been reported^[55,56]. Other reports showed differentiation of bone marrow-derived MAPC toward hepatocyte-like cells induced by FGF-4, however the degree of differentiation was higher when cells were also treated with HGF^[6]. This is consistent with the fact that FGF-4 may play a role in endoderm specification^[47], and that HGF induces differentiation of hepatocytes that are not actively proliferating^[55]. Bone marrow cells cultured with HGF and EGF showed morphologic and phenotypic characteristics of mature hepatocytes^[54]. OSM has been shown to have a specific differentiation-inducing effect on primary fetal hepatic cells towards mature hepatocytes, and the presence of glucocorticoids is required for OSM effects^[49,58].

In this study we have investigated the induction to the hepatogenic differentiation of human ADSC in comparison with BMSC. Seo *et al*^[32] first showed that ADSC can be differentiated into hepatocyte-like cells by treatment with cytokine mixtures (HGF and OSM) and DMSO in serum-free medium. However, we have used a 2-step differentiation protocol with a sequential addition of growth factors (bFGF), cytokines (OSM and HGF), hormones (dexamethasone and insulin) and nicotinamide, which have been reported to be involved in the development and differentiation of hepatocytes^[57,59]. The choice of exogenous factors and the time course to induce hepatogenic transdifferentiation are based on previous reports on BMSC differentiation^[6,39]. As previously mentioned, HGF plays an essential role in the development and regeneration of the liver^[57]; bFGF is required to induce a hepatic fate in the foregut endoderm^[6]; OSM increases hepatocyte size and enhances hepatic differentiation^[57], whereas nicotinamide significantly enhances the *in vitro* maturation of fetal liver cells^[59].

The morphologic and phenotypic features and gene expression changes in both types of cells have been compared. Finally, the role of key hepatic transcription factors in the regulation of the transdifferentiation process has been investigated using adenoviral vectors. The results show that MSCs are capable of giving rise to a hepatogenic transdifferentiation in response to a sequential addition of

growth factors, assessed by an examination of morphology and hepatocyte-specific markers.

It should be highlighted that we have established the culture conditions of both human BMSC and ADSC, as well as the differentiation protocol under adequate conditions for a suitable supply of hepatocyte-like resources for the potential use of human cell transplantation therapy. To achieve this purpose, we have used only a human serum controlled stock from our blood bank instead of fetal calf serum for cell cultures. Cells were serum deprived for 2 d prior to inducing hepatic transdifferentiation, and were then cultured in serum-free conditions (Figure 1). Furthermore, we always used passage 2 cultures for differentiation assays, as it is convenient to differentiate cells for clinical use in low passages to avoid spontaneous differentiation.

Similar morphological changes were observed in BMSC and ADSC, as shown in Figure 4. During Step-1, cells developed a fibroblastic-like flattened shape, but changed to a polygonal morphology during Step-2 in the presence of OMS and hormones. These morphological changes, in parallel with the differentiation process, are coincident with those shown in previous reports either in BMSC^[6,39] or ADSC^[30,32]. BMSC and ADSC cells showed similar expression pattern surface protein markers, and no significant variations of surface marker expression were noted among different samples (Figures 2 and 3). We also found that both undifferentiated MSC cell types were positive for stem cell markers, such as Thy1, CD105 and CD13, but they did not express the hematopoietic marker CD45 in agreement with previous reports^[30,32,58]. The relative amount of Thy1 in undifferentiated ADSC and BMSC cells was significantly higher than in liver cells, but a significant decrease was observed in the differentiated cells, particularly in ADSC. A similar pattern was observed in the amount of AFP in undifferentiated ADSC and BMSC, and it was lower than that in liver. The differentiation potential of BMSCs and ADSC toward the hepatic phenotype was supported by the expression of albumin and CYP isozymes (CYP2E1 and CYP3A4). Albumin expression increased similarly in differentiated BMSCs and ADSC. Homogeneous albumin expression in differentiated ADSC was further confirmed by immunocytochemical analysis, as also shown in BMSC by other authors^[6,32,60]. Concerning the biotransformation capability of differentiated ADSC and BMSC assessed by the expression of CYP2E1 and CYP3A4, a significant increase in CYP2E1 levels was found in differentiated MSCs when compared with the HepG2 cells. The widely used human hepatoma cell line HepG2 expresses gene products that are characteristic of fetal hepatocytes, including serum albumin^[61]. However, hepatoma cells of the fetal phenotype are deficient in the use of certain hepatic transcription factors and show low levels of well-characterized adult liver genes, such as CYP2E1 and CYP3A4. Differentiated ADSC showed higher CYP mRNA levels than HepG2 cells, although the gene expression was still lower than that in the adult liver. Thus, our results suggest that hepatogenic differentiation in ADSC cells was successful, although the hepatocyte adult phenotype was not reached and may require further differentiation steps.

CYP3A4 levels were significantly increased in both differentiated MSCs, although the increase of CYP3A4 levels in differentiated ADSC was consistently higher compared with both the initial time and with HepG2 cells levels. Biotransformation capability, assessed by PROD activity, has been reported in human and rat MAPC derived from bone marrow^[6]. However, studies on ADSC differentiation into hepatic lineage are very scarce^[30,32] and there are no previous results showing CYP expression in differentiated ADSC. The expression pattern and related activity profiles of CYP isoenzymes in the differentiated cells suggest that these cultures might be useful to study many aspects of drug metabolism and drug-related toxicity pathways^[62].

The molecular mechanism, by which our 2-step protocol induces the transdifferentiation of ADSC and BMSC into hepatic lineage cells, seems to be dependent on the induction of several liver-enriched transcription factors, such as C/EBP β and HNF4 α . We opted to investigate these two transcription factors for several reasons. C/EBP β is a key component that distinguishes the liver program of differentiation. The relevance of C/EBP β has been emphasized in recent studies which reveal that pancreatic cells can be transdifferentiated into hepatocytes. In these studies, transdifferentiation was associated with both an elevation of expression of the transcription factor C/EBP β and a reduction of the transcription factor Pdx-1 (pancreatic duodenal homeobox-1). Moreover, transfection of C/EBP β into the cells can provoke transdifferentiation; while a dominant-negative form of C/EBP β can inhibit the process^[63,64]. Similarly, HNF4 α seems to be a very important factor to establish hepatic lineage as HNF4 α was shown to be responsible for the final commitment of oval cells to differentiate into hepatocytes, which regenerate the liver parenchyma^[65]. Moreover, HNF4 α is essential for the morphological and functional differentiation of hepatocytes and the generation of a hepatic epithelium. It has been shown that HNF4 α is a dominant regulator of the epithelial phenotype because its ectopic expression in fibroblasts induces a mesenchymal-to-epithelial transition.

Other liver-enriched transcription factors have also been shown to be associated with the hepatic transdifferentiation of BMSC^[60]. The expressions of HNF1 α , HNF3 α , HNF3 β , HNF4 α , GATA4 and GATA6 were increased during the transdifferentiation of BMSCs, suggesting that hepatic nuclear factors and the GATA family of proteins could also be major components to induce this transdifferentiation^[60]. In this study however, C/EBP α and C/EBP β did not change. Our results demonstrate the induction of C/EBP β during the hepatic transdifferentiation of ADSC and BMSC. This discrepancy in the role of C/EBP β could result from a divergent differentiation protocol. In the study by Saji *et al.*^[60], bFGF was used to induce BMSC transdifferentiation and bFGF may not induce C/EBP β . However, HGF could well be a potent inducer of this transcription factor. It has been shown that HGF induced an early transition of albumin ALB-negative stem cells to ALB-positive hepatic precursors resembling hepatoblasts, and then OMS promoted their differentiation to mature

hepatocytes^[66]. In the first step of stem cell differentiation induced by HGF, the expression of C/EBP β was induced dramatically; when the C/EBP function was inhibited in stem cells, these no longer differentiated to hepatocyte-lineage cells^[66].

In conclusion, the autologous nature of mesenchymal stem cells, together with their putative multipotentiality, may make these cells an excellent choice for many future tissue engineering strategies and cell-based therapies. Previous results indicating the great similarity of the gene expression between BMSC and ADSC by gene array analysis have been reported^[30], supporting the hypothesis that both types of MSC cells originated from a common precursor. They both have a proliferation potential and similar expression pattern of surface markers.

The present study indicates that under certain defined inducing conditions, MSC, isolated from bone marrow and adipose tissue, can differentiate toward a hepatic phenotype *in vitro*. In addition to hepatic biochemical functions, as shown in a previous report^[32], our results demonstrate for the first time that differentiated ADSC also express key drug metabolising enzymes. ADSC have a similar differentiation potential towards the hepatic lineage just as BMSC have, but their abundance and accessibility, their longer culture period and higher proliferation capacity differ from BMSC (as suggested by the present study and previous reports)^[23,30,32]. Therefore, adipose tissue seems to be an ideal source of high large amounts of autologous multilineage mesodermal stem cells for tissue repair and cell therapy of hepatic tissues as well as for preclinical drug testing.

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CLINICAL RESEARCH

Improved method of plasma 8-Isoprostane measurement and association analyses with habitual drinking and smoking

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Abstract

AIM: To develop a simple and accurate method for quantifying 8-isoprostane in plasma by employing a combination of two-step solid-phase extraction of samples and a commercially available ELISA kit, and by this method to examine the effects of drinking and smoking habits against the levels of plasma 8-isoprostane in healthy Japanese volunteers.

METHODS: Plasma 8-isoprostane was extracted with ODS gel suspension followed by NH₂ Sep-Pak column. The 8-isoprostane fractions were assayed using a commercially available ELISA kit. We measured plasma 8-isoprostane levels in 157 healthy Japanese volunteers divided into three groups (64 non-habitual drinkers, 56 moderate drinkers and 37 habitual drinkers) according to their alcohol consumption per week. Genotypes of aldehyde dehydrogenase 2 (ALDH2) were also determined to investigate the plasma 8-isoprostane levels with reference to drinking habits. In addition, the plasma 8-isoprostane levels of 96 non-smokers and 61 smokers from the same subjects were compared.

RESULTS: Our method fulfilled all the requirements for use in routine clinical assays with respect to sensitivity, intra- and inter-assay reproducibility, accuracy and dynamic assay range. Significant increases of plasma 8-isoprostane levels were observed in female habitual drinkers when compared with those of non-habitual drinkers ($t = 5.494$, $P < 0.0001$) as well as moderate drinkers ($t = 3.542$, $P < 0.005$), and 8-isoprostane levels were also significantly different between *ALDH2*2/1* and *ALDH2*1/1* in the female habitual drinkers ($t = 6.930$, $P < 0.0001$), suggesting that excessive drinking of alcohol may increase oxidization stress, especially in females. On the contrary, no significant difference of the plasma 8-isoprostane levels was observed between non-smokers

and smokers.

CONCLUSION: Our present method was proved to be a simple and accurate tool for measuring plasma 8-isoprostane. However, the clinical utility of plasma 8-isoprostane for drinking and smoking habits was limited since elevated 8-isoprostane levels were observed in female heavy drinkers, and no association was found between smokers and nonsmokers.

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Key words: 8-Isoprostane; ELISA; Lipid peroxidation; Drinking; Smoking

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INTRODUCTION

A number of studies have revealed that oxidative stress plays important roles in the pathogenesis of various diseases, such as cancer, diabetes and atherosclerosis^[1,2]. The 8-isoprostane present in biological fluids is produced from arachidonic acid by a non-enzymatic, free radical-catalyzed reaction, and has been proposed as a reliable marker for lipid peroxidation and oxidative stress *in vivo*^[3]. 8-Isoprostane is chemically stable, in contrast to other conventional markers of oxidative stress, and its levels in either plasma or urine are elevated in subjects who smoke^[4-8] and ingest alcohol^[9,10], as well as in patients with diabetes mellitus^[11], heart disease^[12-15], hypertension^[16], preeclampsia^[17] and asthma^[18]. Urinary 8-isoprostane levels increase during the progression of alcohol-induced liver disease^[9] and are decreased by abstinence^[10]. However, accurate measurement of 8-isoprostane is not easy and requires special instruments, such as GC/MS or LC/MS, since various types of analogues and metabolites are present in biological fluids. For instance, the plasma and urinary 8-isoprostane levels determined by a recently developed immunoassay were much higher than those obtained by GC/MS or LC/MS assays^[19,20]. This could be attributed to cross-reaction of the 8-isoprostane analogues and metabolites in the samples with the 8-isoprostane antibody used in the immunoassay.

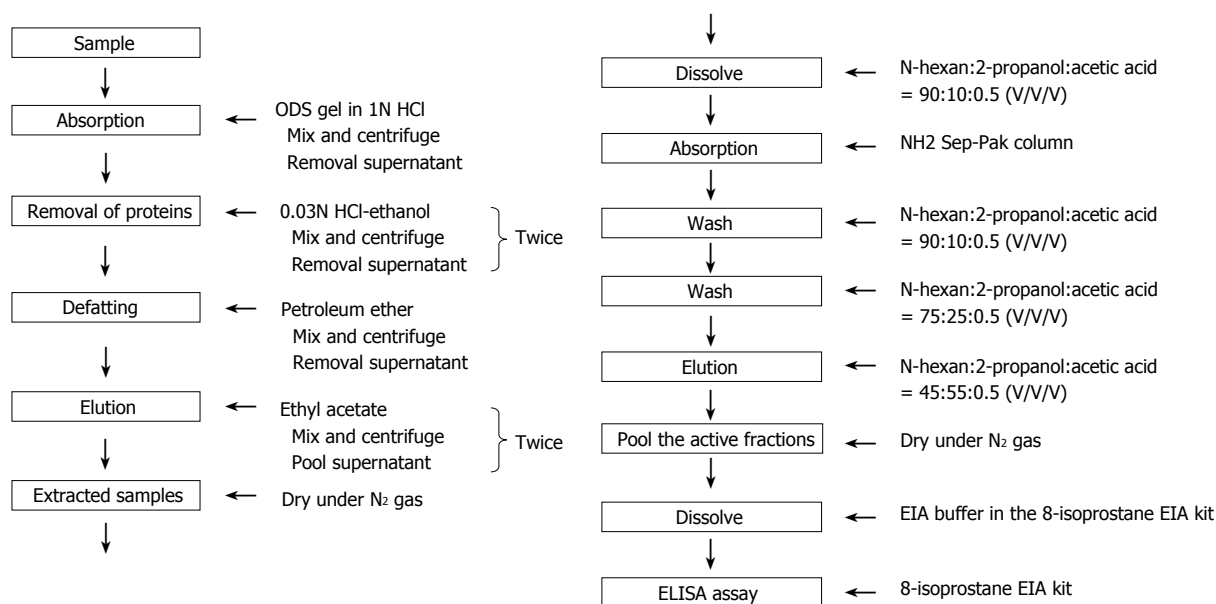


Figure 1 Flow chart of the improved ELISA for plasma 8-isoprostane.

In this study, we developed a new method for pretreatment before analyzing by the commercially available ELISA kit, and performed various examinations for accurate assay of 8-isoprostane. Using this method, we examined the effects of drinking and smoking habits against the levels of plasma 8-isoprostane in healthy Japanese volunteers.

MATERIALS AND METHODS

Samples

After informed consent was obtained, 157 healthy volunteers (83 males and 74 females; age 36.2 ± 8.4 years) were enrolled in this study. The information on drinking and smoking habits was collected by questionnaire. Volunteers were asked the frequency of drinking (nondrinker, rare drinking, 1-2 times/wk, 3-5 times/wk, almost every day), and smoker or nonsmoker. Various lipid parameters (TC, TG, LDL-C, HDL-C, ApoA-I, apoB, apoE and Lp (a)) were measured by EDTA·2Na, and hepatic functions (AST, ALT and γ -GTP) were measured by serum. Blood samples for plasma 8-isoprostane assay were collected in the specific tubes containing 10 mmol/L EDTA·3Na, 20 kU/L Trasylol and 0.1 mmol/L indomethacin, and were separated within 4 h in an ice cooling both. Among the 157 subjects, plasma (heparin) samples were also collected from 3 healthy volunteers to test whether these samples could be used interchangeably for the 8-isoprostane assay. Furthermore, another 3 healthy volunteers as control subjects (2 males, 1 female; age 36.2 ± 8.4 years) were given alcohol (0.5-1.3 g/kg), and their plasma 8-isoprostane, serum AST, ALT, and γ -GTP were measured on D1 (ca. 12 h) and 2 (ca. 36 h) after drinking to investigate the influence of alcohol on these levels. All plasma and serum samples were stored at -80°C until analysis.

Extraction of 8-isoprostane from plasma samples

A two-step solid-phase extraction procedure that was pre-

viously used for quantifying plasma TXA2^[21] was modified for the purification of plasma 8-isoprostane. In principle, the first step of the extraction was performed to remove proteins and lipids using ODS gel (ODS-Q3; Fuji Gel, Tokyo, Japan) and the second step was used to separate 8-isoprostane from its analogues and related compounds using an NH₂ Sep-Pac column (Sep-Pak Vac NH₂; Waters, MA, USA). To optimize the extraction conditions, ³H-labeled 8-isoprostane and other related compounds including PGF2 α , TXB2, 6-keto-PGF1, PGE2 and PGD2 (Cayman Chemical, MI, USA) were used as spiked tracers.

Extraction and measurement of 8-isoprostane

The detailed procedure for plasma 8-isoprostane extraction is shown in Figure 1. One milliliter of ODS gel suspension (80 mg silica gel ODS-Q3 in 0.1 mol/L HCl containing 40 mL/L ethanol) was mixed with 0.5 mL of plasma and allowed to stand at room temperature for 5 min. The gel was collected by centrifugation and washed twice with 1 mL of 0.03 mol/L HCl containing 150 mL/L ethanol and once with 1 mL of petroleum ether to remove proteins and lipids. 8-isoprostane was eluted from the gel twice using 1 mL of ethyl acetate for each elution. The eluates were combined, transferred to another test tube and dried under N₂ gas. The residue containing 8-isoprostane was dissolved in 1 mL of solution A (hexane: 2-propanol: acetic acid = 90:10:0.5, V/V/V), and applied to an NH₂ Sep-Pac column pre-equilibrated with solution A. The column was washed once with 5 mL of solution A, followed by another wash with 5 mL of solution B (hexane: 2-propanol: acetic acid = 75:25:0.5, V/V/V). Finally, 8-isoprostane was eluted from the column with solution C (hexane: 2-propanol: acetic acid = 45:55:0.5, V/V/V) and dried under N₂ gas. The residue was dissolved in 1 mL of the assay buffer included in the 8-isoprostane ELISA kit (Cayman Chemical). The ELISA was performed according to the manufacturer's instructions without further

purification of the samples, and the absorbance was measured with a plate reader (V-Max; Molecular Dynamics, NJ, USA). The 8-isoprostane standards included in the ELISA kit were extracted in the same way as the samples to obtain a calibration curve, which was used to estimate the 8-isoprostane levels in the samples.

Effect of interfering substances on the assay

Interference with the 8-isoprostane assay was tested before and after the addition of free and conjugated-bilirubin (up to 342 $\mu\text{mol/L}$), hemoglobin (up to 5 g/L) and triacylglycerol (up to 55 mmol/L) to each plasma sample. A concentrated reagent set of the interfering substances was purchased from International Reagents Co. Ltd. (Hyogo, Japan).

Storage stability of plasma 8-isoprostane under various conditions

Plasma samples were collected from the 3 control subjects using special tubes as described above. Aliquots of the samples were separately stored at -80°C , 4°C and 25°C . The 8-isoprostane levels were tested within 4 h after the blood collection and on d 1, 3, 7, 14, 21, 28 and 120 using fresh aliquots of the samples at each time point.

Determination of other lipid profiles and hepatic functions in blood samples

The concentration of TC, TG, LDL-C and HDL-C were determined by an enzymatic method (Kyowa Medics Co. Ltd, Tokyo, Japan). ApoA-I, apoB, apoE and Lp (a) were measured by using immunoturbidimetric assay kits (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan). AST and ALT were determined using UV method^[22] and γ -GTP were determined by L- γ -glutamyl-3-carboxy-4-nitroanilid substrate method^[23].

Genotyping of ALDH2

DNA was extracted from blood samples collected with EDTA·2Na using a commercially available kit (Sanko Pure Chemicals Co. Ltd., Tokyo, Japan). Mismatched PCR primers for determining the ALDH2 genotypes were designed with reference to the ALDH2 gene sequence (GenBank Accession No. AH002599). The wild-type allele (*ALDH2*1*) of ALDH2 was amplified using the forward and reverse primers CAAATTACAGGGTCAACTGCTATG and CCACACTCACAGTTTTTCACTTC, respectively. The mutant type (*ALDH2*2*) of ALDH2 was amplified using the forward and reverse primers CAAATTACAGGGTCAACTGCTATG and CCACACTCACAGTTTTTCACTTT, respectively. Amplification was performed in 25 μL of $1 \times$ Qiagen PCR buffer containing 0.2 $\mu\text{mol/L}$ of each ALDH2 primer, 200 $\mu\text{mol/L}$ of each dNTP, and 2 U of HotStartTaq DNA polymerase (Qiagen, Hilden, Germany). The PCR conditions were denaturation at 95°C for 10 min, followed by 35 cycles of amplification (30 s at 94°C , 30 s at 58°C and 30 s at 72°C). After electrophoresis in a 25 g/L agarose gel, the 135-bp PCR products were stained with ethidium bromide and visualized under UV light.

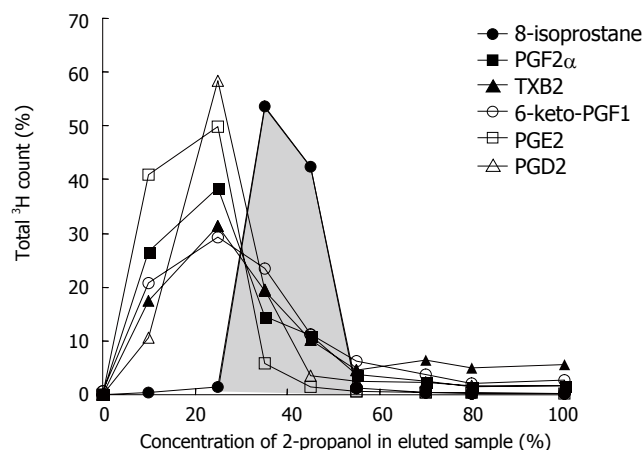


Figure 2 Results of the second extraction step using an NH_2 Sep-Pac column to isolate the plasma 8-isoprostane. Spiked plasma samples containing ^3H -labeled 8-isoprostane, $\text{PGF2}\alpha$, TXB2, 6-keto-PGF1, PGE2 or PGD2 were used. Samples extracted with ODS gel were used to assess the absorption by, washing and elution from the NH_2 Sep-Pac column.

Statistical analysis

Statistical analyses of the data were performed by the paired *t*-test using the In Stat computer software (version 3.06; GraphPad Software Inc.). The correlation between two variables was calculated by the non-parametric Spearman rank coefficient test. A corrected value of $P < 0.05$ was considered statistically significant.

RESULTS

Extraction of plasma 8-isoprostane

Using ^3H -labeled 8-isoprostane and its related compounds as spiked tracers, optimal conditions for the two-step solid-phase extraction of plasma 8-isoprostane were carefully determined. The method was based on a procedure used in a radioimmunoassay for serum TXA2^[21]. First, the plasma samples were treated with reverse-phase ODS gel to remove proteins and lipids. The 8-isoprostane bound to the gel was then eluted and separated from its related compounds on a Sep-Pac NH_2 column by stepwise elution with increasing concentrations of 2-propanol in the eluent. As shown in Figure 2, most of the 8-isoprostane eluted from the column at 55% 2-propanol, whereas the other compounds eluted at 10%-25% 2-propanol. The average yield of plasma 8-isoprostane in the overall extraction was estimated to be 67.1% by counting the ^3H -labeled 8-isoprostane in 5 spiked plasma samples.

Quantification of extracted 8-isoprostane by ELISA

Since it has been shown that plasma 8-isoprostane can be accurately measured using a commercially available ELISA kit after the two-step extraction, we next evaluated the analytical performance and accuracy of the overall ELISA for plasma 8-isoprostane. The detection limit of the ELISA kit was 2.2 ng/L, and this was defined as the concentration corresponding to the optical density of the zero calibrator plus 2SD. The reproducibility of this method estimated using the plasma samples from 3 control subjects was

Table 1 Plasma 8-isoprostane in three groups with different drinking habits (means \pm SD)

	Plasma 8-isoprostane (ng/L)			Group III vs Group I	Group III vs Group I
	Group I	Group II	Group III		
Total	20.3 \pm 6.1 (n = 64)	20.9 \pm 5.7 (n = 56)	26.6 \pm 9.5 (n = 37)	t = 4.059 P < 0.0001 (CI: 3.222-9.387)	t = 3.587 P < 0.001 (CI: 2.523-8.783)
Male	22.5 \pm 6.3 (n = 32)	21.1 \pm 4.9 (n = 31)	23.7 \pm 8.0 (n = 20)	t = 0.589 N.S. (CI: -2.832-5.181)	t = 1.464 N.S. (CI: -0.981-6.252)
Female	18.1 \pm 5.0 (n = 32)	20.8 \pm 6.6 (n = 25)	30.0 \pm 10.3 (n = 17)	t = 5.494 P < 0.0001 (CI: 7.572-16.321)	t = 3.542 P < 0.005 (CI: 3.954-14.466)

CI: 95% Confidence Interval.

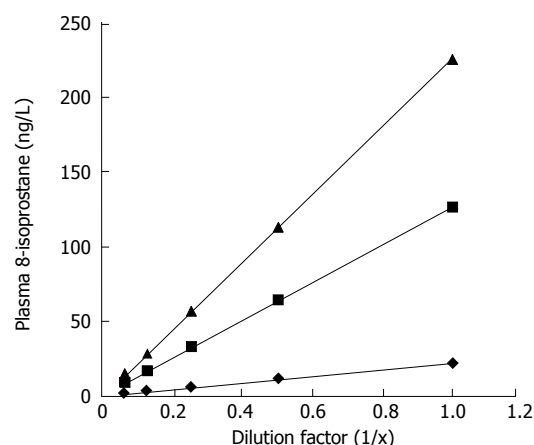
4.2%-6.3% for the within-run (8 repeats) and 0.8%-8.1% for the between-run (5 repeats), respectively. To assess the linearity of the assay, the above mentioned plasma samples were diluted serially, extracted and measured for their 8-isoprostane levels. A good dilution linearity was obtained for the assay, as shown in Figure 3. Analytical recovery studies were carried out using 3 plasma samples containing 3 different concentrations of 8-isoprostane, revealing that the recovery rate ranged from 95.9%-97.8%. There were no significant differences between the 8-isoprostane levels in serum and those of the plasma collected with heparin, EDTA or EDTA + trasirol + indomethacin. All of the samples were freshly prepared and subjected to assay. No interference in the assay was observed for hemoglobin (up to 5 g/L), free bilirubin (up to 342 μ mol/L), conjugated bilirubin (up to 342 μ mol/L) or triacylglycerol (up to 55 mmol/L).

Storage stability of plasma 8-isoprostane

The 8-isoprostane in the plasma samples was stable for at least 120 d at -80°C , as long as freezing and thawing were avoided. However, 2 cycles of freezing at -80°C and thawing at room temperature decreased the apparent 8-isoprostane levels by 30%. It was previously reported that plasma 8-isoprostane was stable for 6 mo at -80°C , and increased approximately 1.4-fold after 3 cycles of freeze and thawing^[4]. Although the reason for this disagreement is not clear, it may be attributed to the method of blood collection, since we used blood collection tubes containing 10 mmol/L EDTA \cdot 3Na, 20 kU/L trasylol and 0.1 mmol/L indomethacin, while they used common evacuated tubes containing EDTA \cdot 2Na (2.5 mmol/L). The EDTA and indomethacin could function to prevent the induction of new synthesis of 8-isoprostane in plasma samples during storage. When the plasma samples were stored at 4°C , 8-isoprostane levels increased rapidly after 1 wk and were 3-4-fold higher after 28 d. When the samples were stored at 25°C , 8-isoprostane levels reached 15-50-fold the original values after 28 d.

Association of plasma 8-isoprostane levels with drinking habits

The mean level of the plasma 8-isoprostane in 157 healthy subjects using our method was 20.9 ± 9.3 ng/L and no age

**Figure 3** Dilution curves of plasma 8-isoprostane in 3 different plasma samples (\blacktriangle $y = 227.6x - 2.34$, $r = 0.999$; \blacksquare $y = 126.3x + 0.06$, $r = 1.000$; \blacklozenge $y = 21.8x - 0.04$, $r = 0.999$).

or gender differences were observed. For the drinking habits obtained by questionnaire, the subjects of each gender were divided into three groups according to their alcohol consumption, namely non-habitual drinkers (nondrinker, rare drinking, Group I, $n = 64$), moderate drinkers (1-2 times drinking/wk, Group II, $n = 56$), and habitual drinkers (3-5 times drinking /wk, Group III, $n = 37$), and the plasma 8-isoprostane levels were compared among these groups. In females, the plasma 8-isoprostane levels were significantly higher in Group III (30.0 ± 10.3 ng/L) than in Group I (18.1 ± 5.0 ng/L, $P < 0.0001$) and II (20.8 ± 6.6 ng/L, $P < 0.005$), but not males (Table 1). The serum γ -GTP levels in Group III were elevated in both genders (males: 488 ± 335 nkat/L at 37°C ; females: 298 ± 185 nkat/L at 37°C) compared with those in Group I (males: 343 ± 253 nkat/L at 37°C ; females: 220 ± 70 nkat/L at 37°C ; $P < 0.05$ each). No significant differences in AST and ALT were observed among the three groups. The plasma 8-isoprostane levels showed no correlations with AST, ALT, γ -GTP and various lipid parameters (TC, TG, LDL-C, HDL-C, ApoA-I, apoB and Lp(a)) in the subjects (Table 2).

Next, the subjects in each group were further divided into 3 groups according to their ALDH2 genotypes, and the plasma 8-isoprostane levels were compared along with those of AST, ALT and γ -GTP. For both the *ALDH2*1/1* and *ALDH2*2/1* genotypes, the plasma 8-isoprostane level was significantly higher in Group III than that in Groups I and II (Table 3) in both genders. This tendency was more prominent in females with the *ALDH2*21/1* genotype. Especially, the 8-isoprostane level was significantly higher in female habitual drinkers with the *ALDH2*2/1* than those with the *ALDH2*1/1* genotype (41.2 ± 12.3 vs 26.9 ± 7.7 ng/L, $P < 0.0001$). The mean level of AST in subjects with the *ALDH2*1/2* genotype was significantly higher in Group III than in Group II (22.3 ± 9.2 vs 17.1 ± 2.5 nkat/L at 37°C , $P < 0.05$). The γ -GTP level was significantly higher in subjects with the *ALDH2*1/1* genotype than those with the *ALDH2*1/2* genotype (478 ± 396 vs 303 ± 198 nkat/L at 37°C , $P < 0.005$). Three healthy subjects (2 males, 1 female) were given alcohol (0.5-1.3 g/kg), and the changes in plasma

Table 2 Spearman correlation coefficients between biochemical parameters ($n = 157$)

	TC	TG	HDL-C	LDL-C	apoA I	apoB	Lp(a)	AST	ALT	γ -GTP	8-isoprostane
Age	0.373	0.122	0.012	0.324	0.088	0.381	0.055	0.116	0.139	0.436	-0.062
TC	-	0.356	0.272	0.818	0.233	0.799	0.211	0.091	0.123	0.273	-0.042
TG	-	-	-0.215	0.446	-0.037	0.565	0.128	0.182	0.247	0.325	-0.020
HDL-C	-	-	-	-0.209	0.849	-0.242	-0.001	-0.068	-0.250	-0.059	-0.101
LDL-C	-	-	-	-	-0.169	0.965	0.264	0.122	0.248	0.235	0.080
apoA I	-	-	-	-	-	-0.155	-0.038	0.022	-0.121	0.083	-0.118
apoB	-	-	-	-	-	-	0.295	0.171	0.296	0.335	0.069
Lp(a)	-	-	-	-	-	-	-	0.057	0.060	-0.029	0.124
AST	-	-	-	-	-	-	-	-	0.897	0.411	-0.034
ALT	-	-	-	-	-	-	-	-	-	0.509	0.002
γ -GTP	-	-	-	-	-	-	-	-	-	-	-0.085

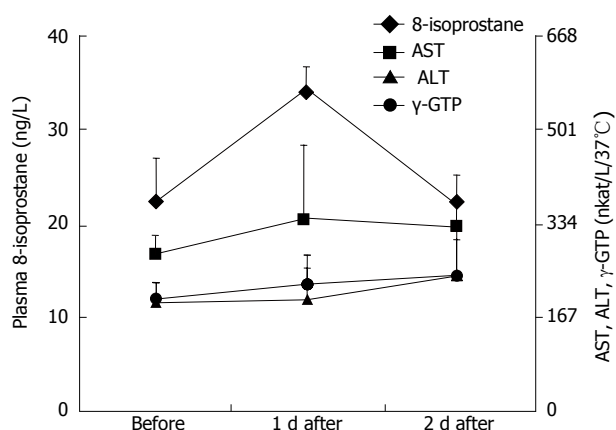


Figure 4 Changes in AST, ALT, γ -GTP and plasma 8-isoprostane after alcohol intake. The levels of plasma 8-isoprostane in the 3 individuals increase significantly on d 1 after drinking, but return to their original levels on d 2. No significant changes are observed in AST, ALT and γ -GTP.

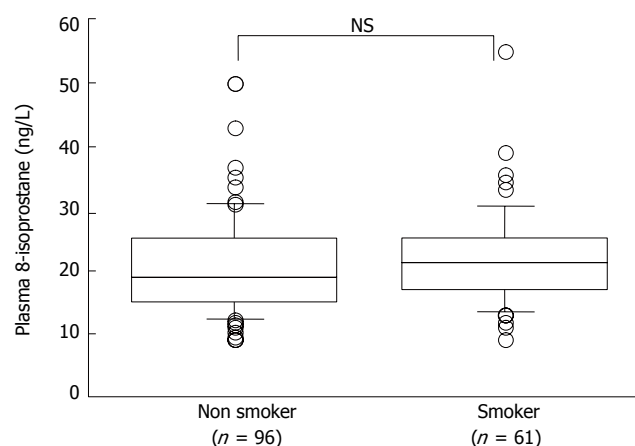


Figure 5 Effect of smoking habit on plasma 8-isoprostane levels. The levels of plasma 8-isoprostane were not significantly different between non smokers and smokers (21.5 ± 7.3 vs 22.8 ± 7.4 ng/L).

8-isoprostane were assayed together with the serum AST, ALT and γ -GTP. Plasma 8-isoprostane significantly increased on d 1, and returned to its original level on d 2 (Figure 4).

Association of plasma 8-isoprostane with smoking habits

The same population of 157 healthy subjects were divided into two groups: non-smokers ($n = 96$; age 36.7 ± 8.2 years) and smokers ($n = 61$; age 35.3 ± 8.7 years), and their plasma 8-isoprostane levels were compared. As shown in Figure 5, no significant difference of plasma 8-isoprostane level was observed between these two groups (21.5 ± 7.3 vs 22.8 ± 7.4 pg/mg).

DISCUSSION

We have developed a simple and accurate method for quantifying plasma 8-isoprostane by employing a combination of two-step solid-phase extraction of samples and a commercially available ELISA kit.

When the plasma 8-isoprostane level was measured by using our method, the mean value of 157 healthy volunteers was 20.9 ± 9.3 ng/L, which is almost equal to the reported values using GC/MS or LC/MS^[5,24,25]. The level of plasma 8-isoprostane in 4 healthy subjects among these samples measured directly by ELISA without

extraction was more than 20-fold higher than those obtained by the combination of the two-step extraction and ELISA (651.5 ± 149.2 vs 24.4 ± 4.1 ng/L). According to 8-Isoprostane EIA kit booklet, although the cross-reactivity of the anti 8-isoprostane antibody employed in the ELISA kit is reported to be low, the extraction of plasma 8-isoprostane from plasma prior to the assay is indispensable. In fact, it has been reported that various 8-isoprostane analogues and related compounds are present at dozens to hundreds of times than the concentration of 8-isoprostane in biological fluids^[26].

In an association study of plasma 8-isoprostane with drinking habits, heavy drinkers were higher than non-habitual drinkers and moderate drinkers. However, the plasma 8-isoprostane level was not significantly different when compared among the 3 male groups. Also, the plasma 8-isoprostane levels were significantly higher in heavy drinkers than in non-habitual and moderate drinkers. This tendency was more prominent in females, and with the *ALDH2*21/1* genotype. In other words, the 8-isoprostane level was significantly higher in female habitual drinkers with the *ALDH2*2/1* than with the *ALDH2*1/1* genotype. These results suggest that excessive drinking may increase oxidative stress, especially in females with the *ALDH2*2/1* genotype. When the same amount of alcohol is ingested, females tend to show

Table 3 Plasma 8-isoprostane analyzed by ALDH2 genotype in three groups with different drinking habits (means \pm SD)

		Plasma 8-isoprostane (ng/L)			Group III <i>vs</i> Group I	Group III <i>vs</i> Group I
		Group I	Group II	Group III		
ALDH2*1/1	Male	20.2 \pm 5.8	19.8 \pm 5.0	22.8 \pm 5.4	t = 1.165, N.S. (CI: -1.988-7.137)	t = 1.664, N.S. (CI: -0.684-6.758)
	Female	19.0 \pm 5.8	21.6 \pm 6.6	26.9 \pm 7.7	t = 2.754, P < 0.05 (CI: 1.926-13.808)	t = 1.897, N.S. (CI: -0.447-10.887)
	Total	19.6 \pm 5.7 (n = 22)	20.6 \pm 5.8 (n = 33)	24.6 \pm 6.7 (n = 27)	t = 2.776, P < 0.01 (CI: 1.375-8.616)	t = 2.471, P < 0.05 (CI: 0.757-7.219)
ALDH2*1/2	Male	23.7 \pm 7.5 (n = 11)	22.6 \pm 4.4 (n = 12)	28.5 \pm 14.8 (n = 4)	t = 0.834, N.S. (CI: -7.517-16.976)	t = 1.281, N.S. (CI: -3.934-15.600)
	Female	16.0 \pm 4.0 (n = 15)	19.9 \pm 7.4 (n = 8)	41.2 \pm 12.3 (n = 3)	t = 6.930, P < 0.0001 (CI: 17.459-32.848)	t = 3.622, P < 0.01 (CI: 8.017-34.683)
	Total	19.3 \pm 6.8 (n = 26)	21.5 \pm 5.8 (n = 20)	33.9 \pm 14.4 (n = 7)	t = 3.898, P < 0.001 (CI: 6.973-22.276)	t = 3.267, P < 0.005 (CI: 4.586-20.221)
ALDH2*2/2	Male	22.2 \pm 5.5 (n = 6)	-	-	-	-
	Female	21.8 \pm 2.0 (n = 3)	-	-	-	-
	Total	22.0 \pm 4.7 (n = 9)	-	-	-	-

CI: 95% confidence interval.

a higher concentration of blood alcohol than males due to their lower body weight and higher ratio of adipose tissue into which alcohol shows poor penetration. In addition, female hormones, such as estradiol, inhibit the activity of ADH, contributing to the increased concentration of blood alcohol^[27].

Changes in plasma 8-isoprostane levels after alcohol (0.5-1.3 g/kg) intake for 3 d, showed 8-isoprostane significantly increased on d 1, and returned to its original level on d 2. It was previously reported that alcohol consumption induced lipid peroxidation in healthy volunteers, and urinary 8-isoprostane increased in a dose- and time-dependent manner reaching its peak at 0-6 h after ingestion^[9], and through induction of the CYP450 2E1 isozyme, alcohol intake may increase the generation of reactive oxygen intermediates that have the potential to peroxidize lipids^[28,29]. Similarly, our results may suggest that alcohol ingestion induced oxidative stress in a relatively short time after drinking.

It has been reported that smoking induces oxidative stress and increases urinary 8-isoprostane^[30]. Therefore, we compared plasma 8-isoprostane levels in non-smokers and smokers. In contrast to urinary 8-isoprostane, no difference in plasma 8-isoprostane was observed between these 2 groups. This is consistent with a previous report measured by LC/MS^[5]. The plasma 8-isoprostane may be rapidly metabolized since 75% of plasma 8-isoprostane is excreted in the urine within 4.5 h^[31].

In conclusion, we have developed a simple and accurate method for quantifying plasma 8-isoprostane by employing a combination of two-step solid-phase extraction of samples and a commercially available ELISA kit. Our method fulfilled all the requirements for use in routine clinical assays with respect to sensitivity, intra- and inter-assay reproducibility, accuracy and dynamic assay range. However, the clinical utility of plasma 8-isoprostane for drinking and smoking habits was limited. Further studies

using a large population is required for a final conclusion for an association of plasma 8-isoprostane with drinking and smoking habits.

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Doppler study of hepatic vein in cirrhotic patients: Correlation with liver dysfunction and hepatic hemodynamics

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Abstract

AIM: To elucidate the significance of Doppler measurements of hepatic vein in cirrhotic patients and to correlate with liver dysfunction and hepatic hemodynamics.

METHODS: One hundred patients with liver cirrhosis and 60 non-cirrhotic controls were studied. Doppler waveforms were obtained from right hepatic vein and flow velocity measured during quiet respiration. Doppler measurements were also obtained from portal trunk, right portal vein and proper hepatic artery.

RESULTS: Hepatic vein waveforms were classified into three classical patterns. Flat waveform was uncommon. Mean hepatic vein velocity was significantly higher in cirrhotic patients (12.7 ± 6.4 vs 5.1 ± 2.1 and 6.2 ± 3.2 cm/s; $P < 0.0001$). The poorer the grade of cirrhosis, the higher was the mean velocity. Maximum forward velocity was never greater than 40 cm/s in controls. Degree of ascites was found to be highly correlated with mean velocity. "Very high" group (≥ 20 cm/s) presented clinically with moderate to massive ascites. Correlations between right portal flow and mean velocity was significant ($P < 0.0001$, $r = 0.687$).

CONCLUSION: Doppler waveforms of hepatic vein, which is independent of liver dysfunction, should be obtained during normal respiration. Mean hepatic vein velocity reflects the change in hepatic circulation associated with progression of liver cirrhosis. It can be used as a new parameter in the assessment of liver cirrhosis.

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Key words: Hepatic vein; Hepatic vein velocity; Doppler ultrasound

INTRODUCTION

There are many studies on inflow to the liver in liver cirrhosis (LC) in relation to hepatic dysfunction and portal hypertension. In LC, there are changes in liver parenchyma as well as alteration of hepatic vasculature, including morphological changes of the hepatic vein. Furthermore, it is accepted that abnormal circulation caused by hyperdynamic circulation and intrahepatic shunt persists in cirrhotic liver. Therefore, we assumed that an alteration in hepatic venous hemodynamics is present in cirrhotic liver. It is of interest to find out the clinical significance of hepatic venous hemodynamic changes in liver cirrhosis, and to explore which factor, singly or in combination, produces such changes if there is any. Due to the fact that hemodynamic studies of the hepatic vein is still limited, we aimed to study the hepatic venous hemodynamics using non-invasive Doppler ultrasonography.

Doppler waveform of the hepatic vein (HV) in healthy humans is a triphasic waveform, consisting of two negative waves and one positive wave^[1]. As for abnormal hemodynamics of the hepatic vein in cirrhotic liver, there are reports of Doppler studies suggesting that the waveform becomes flat when hepatocellular function is impaired, and some studies even proposed that flattening of the hepatic waveform could be used as a diagnostic tool for chronic parenchymal liver disease^[2-6]. However, there are equivocal results regarding change in waveforms of the HV in parenchymal liver diseases. The large degree of overlap in the waveforms between various types of parenchymal liver diseases makes the interpretation of waveform difficult. In this study, we aimed to reevaluate the use of Doppler waveform of the right hepatic vein in LC without respiratory maneuver, as there have been conflicting reports on use of it as a diagnostic tool in the chronic parenchymal liver disease. Furthermore, we studied a quantitative analysis of hepatic venous blood flow, which, to the best of our knowledge, has not been reported previously.

MATERIALS AND METHODS

Subjects

One hundred and sixteen consecutive patients with LC, subjected to Doppler examination from July 2002 to September 2003, were enrolled in this prospectively designed study. Patients with enlarged inferior right hepatic vein ($n = 2$), portal thrombosis ($n = 5$), hepatofugal portal blood flow ($n = 2$), tricuspid regurgitation ($n = 2$), insufficient visualization of hepatic artery ($n = 2$), right portal vein ($n = 1$), and right hepatic vein ($n = 2$) were excluded from the study. Remaining 100 patients (57 women and 43 men; mean age 63 ± 7 years) were studied. The diagnosis of LC was based on histopathology and/or imaging diagnosis along with clinical and biochemistry parameters. The etiology of LC was viral in 77 (HCV: 69; HBV: 7; HCV + HBV: 1), alcohol in 14 and primary biliary cirrhosis in 9 patients. The severity of liver dysfunction, as assessed by Child-Pugh scoring system^[7], was A in 46, B in 29 and C in 25 patients. In addition, study was carried out in randomly selected 60 non-cirrhotic patients as controls. Among them were 30 patients with histopathological diagnosis of chronic hepatitis (14 women and 16 men; mean age 57 ± 7 years) without any evidence of LC and same number of volunteers (17 women and 13 men; mean age 57 ± 6 years) without any clinical, laboratory or imaging evidence of liver disease. Causes of chronic hepatitis were hepatitis C virus in 24, hepatitis B virus in 6 patients. The control group did not differ significantly from the study population with respect to age, sex, height and weight. Cardiac disease and respiratory disease that may cause change in the Doppler waveform of the hepatic vein were ruled out in all patients and controls.

Instrument

The instrument used in this study was a pulsed Doppler flowmeter (PowerVision 8000) with a 3.75-MHz convex and sector probe. The system is equipped with software to compute the time-averaged velocity from the velocity spectral display after placement of the calipers. Doppler sample volume was positioned in the center of the vessel or at the point when it was defined. The sample width was selected to cover almost entire vessel diameter. Pulse repetition frequency was adjusted so as not to exceed the limit of the displayed maximum velocity. Care was taken to ensure that the angle of insonation was always smaller than 60. Internal diameter of the vessels was measured manually after optimizing B mode images.

Doppler measurements

Each subject was examined after overnight fasting in supine position after a rest of 15 min so as to avoid any influence of food, posture and exercise. Overall assessment of the hepatobiliary system by B mode was made before Doppler examination. Size of the right lobe in cirrhotic patients was assessed by the method described by Matsutani *et al*^[8]. Visual grading of ascites as appeared during ultrasonography was done. Right hepatic vein was approached from right intercostal space. The reason behind choosing the right hepatic vein over other is that the middle and left hepatic vein often join each other before draining to the inferior venacava. Doppler study

of the hepatic vein was performed by positioning the sample volume between 2-3 cm from the opening into the inferior venacava. Patients were asked to breath normally during tracing of the waveforms. Hepatic Doppler waveforms were divided into three types. The waveforms were considered triphasic when one of the waves showed reversed flow, that is, any wave above the baseline. Flat waveforms were those without any phasic oscillation. Those without reversed flow but showing phasic oscillations were regarded as biphasic waveform. Mean flow velocity was calculated automatically by the instrument after tracing of the spectral display (of one cardiac cycle), whereas maximum and minimum velocity were calculated manually. For flat waveforms, ECG was simultaneously recorded to determine one complete cardiac cycle.

The portal vein was evaluated at about middle of the portal trunk by approaching from the epigastrium with the probe placed slightly obliquely. The right portal vein was approached from the right intercostal spaces. Mean velocity and flow (of portal trunk and right portal vein) were calculated by instrument after obtaining Doppler waveforms. The hepatic artery was examined similarly to the portal trunk as it runs almost parallel with the portal vein before entering into the hepatic hilum. At this position gastroduodenal arteries are expected to have branched from the common hepatic artery. Peak systolic velocity was calculated manually, whereas mean velocity was calculated by the instrument after manual tracing of the maximum point of the spectral display. Doppler examinations were performed by two authors (SKC and SM) without prior knowledge of clinical and biochemical status of the study population. Three readings were taken for each vessel and the mean of three was noted as the final reading. Written informed consent was obtained from all the patients and controls. The study protocol conforms to the ethical guidelines of the 1975 Helsinki Declaration, and approved by Senior Staff Committee.

Statistical analysis

The statistical analyses were carried out using SPSS ver. 10.0 (SPSS Inc., Chicago, IL, USA). All the measurements were expressed as mean \pm SD. To compare means, Student's *t* test was performed. For multiple values, ANOVA (analysis of variance) with LSD (least square design) was used. $P < 0.05$ and $r > 0.6$ were considered statistically significant.

RESULTS

Hepatic vein waveforms

All non-cirrhotic controls showed triphasic waveforms of HV. In cirrhotic patients triphasic pattern was observed in 49 (49%) patients, biphasic in 48 (48%) patients and flat in 3 (3%) patients. Types of the waveforms according to the grade of liver function are shown in Table 1. The result showed that the appearance of waveform was independent of the degree of severity of liver dysfunction as graded by Child-Pugh score. Flat waveform was very rare, appearing only on three occasions. Out of 3 patients, 2 were Child-Pugh A and were clinically stable. Hepatic encephalopathy and ascites were absent in all 3 patients.

Table 1 Hepatic vein waveforms in study population *n* (%)

	Non-cirrhotic controls		Liver cirrhosis patients		
	No liver pathology	Chronic hepatitis	A <i>n</i> = 46	B <i>n</i> = 29	C <i>n</i> = 25
Triphasic	30	30	26 (56.6%)	16 (55.2%)	7 (28%)
Biphasic	0	0	18 (39.1%)	13 (44.8%)	17 (68%)
Flat	0	0	2 (4.3%)	0	1 (4%)

There was no correlation between waveforms and liver dysfunction.

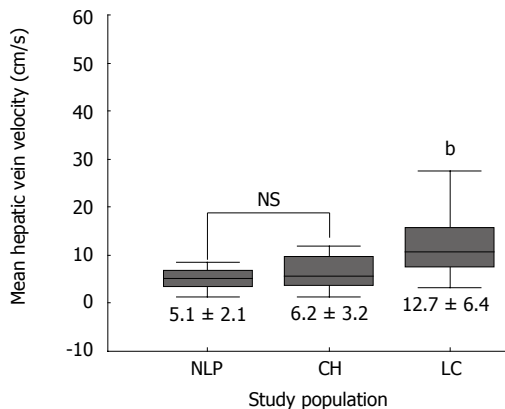


Figure 1 Mean hepatic vein velocity in study populations. It was significantly higher in cirrhotic patients than non-cirrhotic controls. (mean \pm SD; $^bP < 0.0001$ vs NLP and CH).

Correlation of hepatic vein velocity with liver dysfunction

Table 2 summarizes the results of hemodynamic data in the study population. Mean hepatic vein velocity was significantly higher in cirrhotics than non-cirrhotic controls (12.7 ± 6.4 vs 5.1 ± 2.1 and 6.2 ± 3.2 cm/s; $P < 0.001$) (Figure 1). There was no statistical significance of mean among three etiological subsets of cirrhotic patients (data not shown). The poorer the grade of cirrhosis by Child-Pugh grade, the higher was the velocity (Figure 2). Rise in MHVV was independent of hepatic vein diameter. We defined the “very high” group as those patients who had MHVV 20 cm/s or more. We took watershed of 20 cm/s as it was more than mean plus SD values and provided best separation between two groups. There were 15 patients who had MHVV more than 20 cm/s. Similarly, maximum forward velocity was higher in LC patients than controls (Figure 3). It was never more than 40 cm/s in controls. There was no correlation of MHVV with serum bilirubin ($P < 0.11$, $r = 0.279$), serum albumin ($P < 0.12$, $r = -0.276$) and prothrombin time ($P < 0.002$, $r = -0.377$) (Figure 4 A-C). On the other hand, there was strong correlation of MHVV with grades of ascites. We divided the patients into three groups: nil ($n = 62$), minimal ($n = 16$) and moderate to massive ($n = 22$), depending upon the accumulation of ascitic fluid. Cases associated with moderate to massive ascites had significantly higher MHVV than other groups (Figure 5). Correspondingly, “very high” group were always associated with moderate to massive ascites.

Correlation of hepatic vein velocity with hepatic hemodynamics

There was no correlation of MHVV with portal flow,

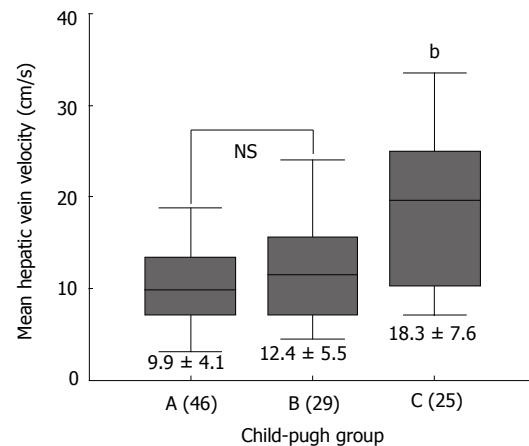


Figure 2 Mean hepatic vein velocity in study population. Hepatic vein velocity in cirrhotic patients according to Child-Pugh group (mean \pm SD; $^bP < 0.0001$ vs A and B).

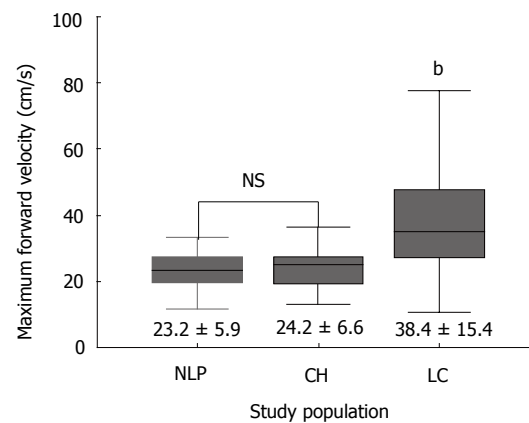


Figure 3 Maximum forward velocity of hepatic vein in study populations. (mean \pm SD; $^bP < 0.0001$ vs NLP and CH).

portal velocity and hepatic artery velocity ($r = -0.105$, 0.391 and 0.297 , respectively). However, we found a significant correlation of MHVV with right portal flow in LC patients ($P < 0.0001$, $r = 0.687$) (Figure 6A). No such correlation was seen in non-cirrhotic controls ($P < 0.767$, $r = -0.042$) (Figure 6B). Right portal flow was significantly higher in the “very high” group (331 ± 120 mL/min vs 526 ± 133 mL/min) (Figure 7). Those cases, which were not associated with increased right portal flow, had increased peak systolic velocity of the hepatic artery in the “very high” group.

The intra-observer variability was 5% in the hepatic vein, 7% in the portal trunk and right portal vein and 10% in the hepatic artery measurements. Regarding tracing of waveform of right hepatic vein, it was nil.

DISCUSSION

This study argues against the appearance of flat waveform of the hepatic vein in cirrhotic patients with impaired liver function. This is based on the finding that the waveform of the hepatic vein in this study was independent of liver function and Child-Pugh score. Only 3 patients showed flat waveform and only 1 of them was C by Child-Pugh grading. However, change of waveform to biphasic from

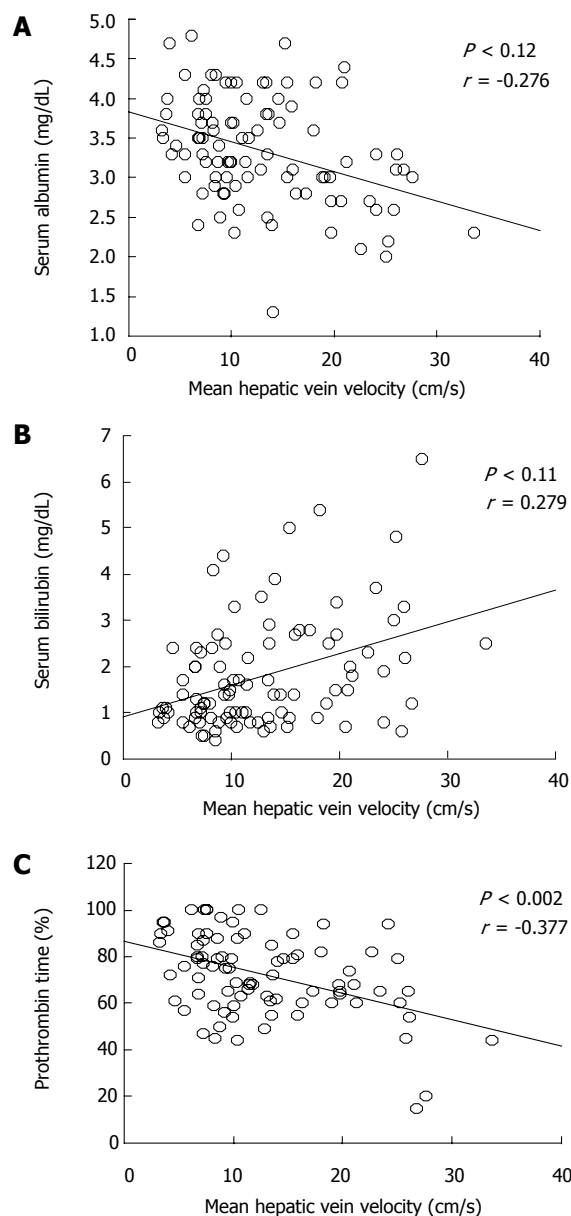


Figure 4 Relation between mean hepatic vein velocity and liver function test in cirrhotic patients. **A:** serum albumin; **B:** serum bilirubin; **C:** prothrombin time.

triphasic seems suggestive of some changes in the liver parenchyma as only cirrhotic patients showed change from triphasic to biphasic or flat waveform. Although the phasicity is cardiac in origin, predominantly the change in right atrial pressure changes, respiratory motion can alter the HV waveforms and its components^[9]. We noticed that the negative oscillation disappears with respiratory maneuvers in some patients and this may be the reason for discrepancy in findings regarding the negative waves in many studies. However, this change in waveforms with different respiratory maneuvers was an equivocal finding and the reason is not well understood why it occurs in some patients only. In the same line, flat waveform in 9.33% (7/75) subjects without liver and cardiac disease can be explained^[10]. Bolondi *et al*^[2] have reported that the underlying mechanism of the change in the hepatic vein waveform may be related to liver fibrosis, which progressively reduces phasic oscillation in hepatic veins. However, in our patients, triphasic waveform was seen

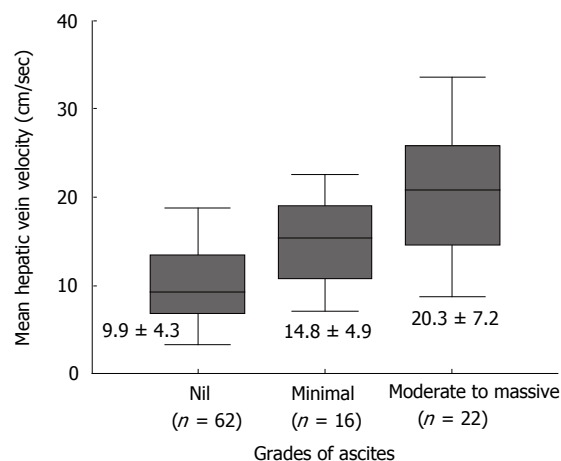


Figure 5 Mean hepatic vein velocity in patients depending upon the accumulation of ascites. (mean \pm SD; ^b $P < 0.01$ vs nil and moderate to massive).

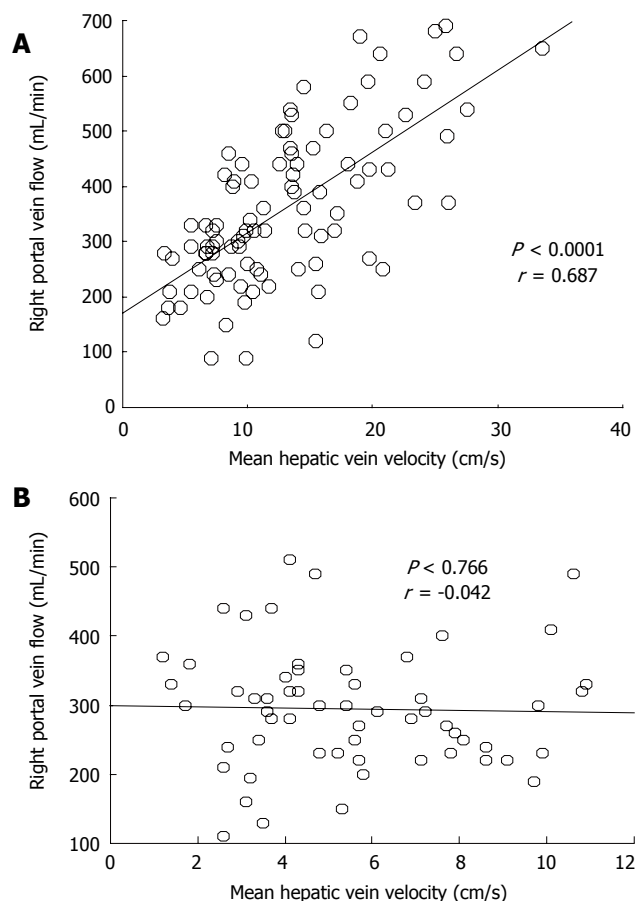


Figure 6 Correlation between mean hepatic vein velocity and right portal vein flow in cirrhotic patients (**A**) and controls (**B**).

even in significantly atrophied liver with marked nodule formation on histopathological specimens. Our experience showed that inadvertent measurement of Doppler waveform from inferior right hepatic vein or enlarged marginal vein sometimes might give flat waveform. In two of the cases that were excluded in this study, flat waveforms were obtained from the inferior right hepatic vein, while right hepatic vein showed triphasic waveforms.

To our knowledge, this is the first time MHVV has

Table 2 Hemodynamic data in study population (mean \pm SD)

Hemodynamic	Control (non-cirrhotic)		Liver cirrhosis		
	NLP	CH	A	B	C
Portal trunk					
Diameter (mm)	8.9 \pm 1.0	9.9 \pm 1.0	11.9 \pm 2.5	11.6 \pm 2.0	12.5 \pm 2.5
MFV (cm/s)	13.3 \pm 3.1	11.5 \pm 3.1	9.96 \pm 2.91	9.40 \pm 2.41	9.59 \pm 2.54
FV (mL/min)	516 \pm 96	504 \pm 140	691 \pm 323	700 \pm 226	753 \pm 206
Right Portal vein					
Diameter (mm)	7.5 \pm 1.0	7.6 \pm 1.0	9.1 \pm 1.9	9.6 \pm 2.0	9.7 \pm 1.7
MFV (cm/s)	12.2 \pm 3.7	11.2 \pm 2.8	8.45 \pm 2.97	8.31 \pm 2.43	9.31 \pm 2.01
FV (mL/min)	318 \pm 92	282 \pm 86	357 \pm 182	365 \pm 139	415 \pm 169
Proper hepatic artery					
Diameter (mm)	2.8 \pm 0.3	3.1 \pm 0.5	3.6 \pm 0.7	3.6 \pm 0.7	3.8 \pm 0.8
MFV (cm/s)	26.9 \pm 5.1	24.4 \pm 5.5	25.0 \pm 10.8	26.3 \pm 6.3	28.8 \pm 11.3
V _{max} (cm/s)	55.2 \pm 12.0	55.3 \pm 11.4	56.3 \pm 18.5	61.1 \pm 16.9	64.4 \pm 21.8
Hepatic vein					
Diameter (mm)	5.8 \pm 1.6	6.0 \pm 1.1	5.5 \pm 1.6	4.7 \pm 1.1	4.7 \pm 1.1
MHVV (cm/s)	5.1 \pm 2.1	6.6 \pm 3.2	9.7 \pm 4.1	11.6 \pm 5.4	19.1 \pm 10.3
MFFV (cm/s)	23.1 \pm 6.0	24.7 \pm 6.3	31.8 \pm 11.4	40.2 \pm 16.4	48.8 \pm 17.5

CH: Chronic hepatitis; NLP: No liver pathology; MHVV: Mean hepatic vein velocity; MFV: Mean flow velocity; FV: Flow volume; V_{max}: Peak systolic velocity; MFFV: Maximum forward flow velocity.

been measured by Doppler in cirrhotic patients. It was significantly higher in cirrhotic than non-cirrhotic patients and there was significant difference of mean between cirrhotic and non-cirrhotic controls. Furthermore, the poorer the liver function, the higher was the MHVV. No increase in MHVV in chronic hepatitis patients indicates that no measurable hemodynamic changes occur before the onset of LC. On line with other studies^[4], maximum forward velocity was also significantly elevated in LC patients. In non-cirrhotic patients, the maximum velocity was never higher than 40 cm/s. Thus, we propose that coupled with maximum forward velocity, it can be used as a supplementary diagnostic tool in liver cirrhosis. As there was no correlation between serum albumin, serum bilirubin, prothrombin time, role of liver cell dysfunction was ruled out. However, ascites showed significant correlation with MHVV which was significantly higher in the patients presenting clinically with moderate to massive ascites. Thus, we hypothesize that the MHVV is subjected to change with hepatic hemodynamics not directly related to liver cell function.

A positive correlation of hepatic vein velocity with grades of ascites was found. Although authentic volumetric data was not obtained in study cases, those cases with very high velocity had atrophied right lobe of the liver on ultrasonography (data not included). Our finding is also supported by Torres *et al*^[11], who have reported that in LC, the right lobe frequently atrophies, while the left lateral lobe becomes hypertrophic. High inflow of the atrophied right lobe of the liver may be one of the plausible explanations of relation with ascites. In an *in vitro* liver perfusion system, lymph could be seen oozing from the surface of the liver when portal perfusion pressure was acutely increased^[12]. However, further study is considered necessary to establish the exact role of hepatic microcirculation, which appears to be instrumental in modifying MHVV, with ascites formation, as it is still

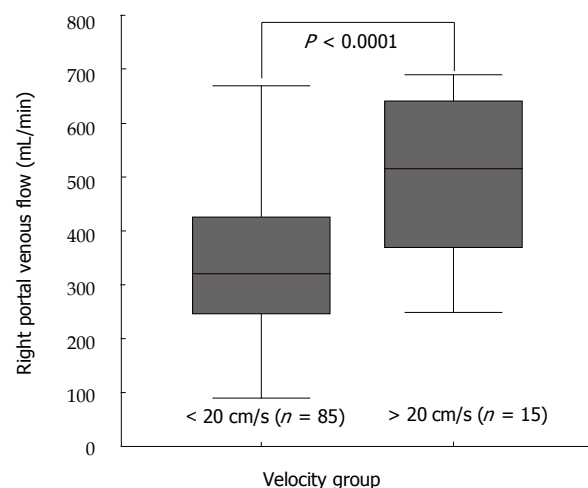


Figure 7 Right portal flow in cirrhotic patients. "Very high" group, which we defined as those having velocity \geq 20 cm/s had increased perfusion of right lobe of the liver.

imprecisely known. If the mechanism is uncovered, measurement of MHVV can be used to evaluate the efficacy of different treatment modalities in the treatment of ascites related to LC.

In this study, cirrhotic patients showed correlation of MHVV with right portal flow, while non-cirrhotic controls demonstrated no correlation between them. In normal subjects, blood flows from the portal venules into the hepatic vein system traversing through hepatic sinusoids. An autoregulation of flow at the level of sinusoids is present in normal liver, which is supposed to control the blood flowing to the hepatic vein. However, in the cirrhotic liver, there are various changes. It is reported that a direct communication occurs between two venous systems^[13]. Moreover, it has been hypothesized that functionally inactive pre-existing arterio-venous shunts are opened in a response to intense vasodilatation^[14]. Hiraki *et al*^[15] showed

that maximum peak velocity of right portal vein decreased substantially with temporary occlusion of the right hepatic vein. Change in hepatic arterial hemodynamics was seen with the same maneuver. Therefore, the alteration in hemodynamics of the portal vein and hepatic artery can have an influence on hepatic vein, its flow and waveform. Our study also proves this fact as those cases associated with high velocity of the right hepatic vein were found to have increased perfusion of the right lobe of the liver by right portal vein. It can be explained by the fact that there is increased porto-venous shunt in cirrhotic liver and the flow may have bypassed to the hepatic vein, thereby increasing its flow velocity. In cases not associated with increased right portal flow, hepatic artery velocity was increased. Arterial flow is expected to increase in LC due to arterIALIZATION of the cirrhotic liver that contributes to increased inflow to the liver^[16]. Furthermore, there is arterio-venous shunt formation in cirrhotic liver that usually can not be detected, even by standard imaging diagnostic procedures. In recent years, transit-time analysis of contrast agent in cirrhotic patients has been proposed for non-invasive diagnosis of LC^[17]. The findings have shown that there is early arrival time of the contrast agent and early peak enhancement in HV of cirrhotic patients compared to non-cirrhotic patients. Similar study carried out recently concluded that intrahepatic hemodynamic changes are responsible for this^[18]. This supports the result of our findings. However, there is deranged autoregulation of sinusoidal flow present in cirrhotic liver along with intrahepatic shunt formation; it is difficult to arbitrate which factor is more predominant to determine the correlation.

In vitro study has shown that Doppler flowmetry is relatively accurate and reproducible. Thus, this technique is increasingly being used in clinical practice to study hepatic hemodynamics. We have applied it to study the right hepatic vein and an attempt has been made to correlate the hemodynamic findings with severity of liver dysfunction in cirrhotic patients. We observed that the cirrhotic patients had increased velocity of the hepatic vein and those cases with very high velocity were having poorer liver function as graded by Child-Pugh classification. Correlation of MHVV with ascites, but not albumin, bilirubin or prothrombin time substantiates its association with hepatic microcirculation. Simple measurement of hepatic vein velocity can give information on deranged hepatic microcirculation. Hence, we advocate that Doppler measurement of hepatic vein velocity, which is non-invasive, can be used as a new parameter in the investigation of chronic parenchymal liver disease.

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Acute interstitial edematous pancreatitis: Findings on non-enhanced MR imaging

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weighted images were, respectively, 85% for pancreatic fascial plane, 77% for left renal fascial plane, 55% for peripancreatic fat stranding, 42% for right renal fascial plane, 45% for perivascular fluid, 40% for thickened pancreatic lobular septum and 25% for peripancreatic fluid, which were markedly higher than those on in-phase or SSFSE T2-weighted images ($P < 0.001$).

CONCLUSION: IEP primarily manifests on non-enhanced MR images as thickened pancreatic fascial plane, left renal fascial plane, peripancreatic fat stranding, and peripancreatic fluid. R-T T2-weighted imaging is more sensitive than in-phase and SSFSE T2-weighted imaging for depicting IEP.

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Key words: Pancreas; Pancreatitis; Inflammation; Edema; Magnetic resonance

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Abstract

AIM: To study the appearances of acute interstitial edematous pancreatitis (IEP) on non-enhanced MR imaging.

METHODS: A total of 53 patients with IEP diagnosed by clinical features and laboratory findings were underwent MR imaging. MR imaging sequences included fast spoiled gradient echo (FSPGR) fat saturation axial T1-weighted imaging, gradient echo T1-weighted (in phase), single shot fast spin echo (SSFSE) T2-weighted, respiratory triggered (R-T) T2-weighted with fat saturation, and MR cholangiopancreatography. Using the MR severity score index, pancreatitis was graded as mild (0-2 points), moderate (3-6 points) and severe (7-10 points).

RESULTS: Among the 53 patients, IEP was graded as mild in 37 patients and as moderate in 16 patients. Forty-seven of 53 (89%) patients had at least one abnormality on MR images. Pancreas was hypointense relative to liver on FSPGR T1-weighted images in 18.9% of patients, and hyperintense in 25% and 30% on SSFSE T2-weighted and R-T T2-weighted images, respectively. The prevalences of the findings of IEP on R-T T2-

INTRODUCTION

Acute pancreatitis (AP) is a protean disease of wide clinical variation ranging from mild discomfort to severe multiorgan failure and death. Acute interstitial edematous pancreatitis (IEP), found in approximately 75% of patients presenting with AP, consists of edema and inflammation of the pancreas and is typically a self-limiting process with a mild clinical course^[1,2]. Necrotizing pancreatitis (NP) is a far more severe form of pancreatitis, which is characterized by extensive fat necrosis, hemorrhage, and necrotic liquefaction of the pancreas. Dynamic CT is well established for differentiating IEP from NP^[3]. However, CT is not necessary in depicting IEP or mild acute pancreatitis^[2,4,5]. Patients with AP are often young and require multiple follow-up CT examinations; substitution of MRI for CT in some patients would reduce their collective radiation dose considerably. In addition, experimental study^[6] and clinical retrospective analysis^[7]

of pancreatitis suggested that iodinated contrast medium might worsen or prolong attacks of acute pancreatitis.

Recent advances in abdominal MRI improve the spatial resolution of images and allow optimal imaging of the pancreas. MRI can have an important role in staging the severity of acute pancreatitis and may be superior to other imaging techniques for the characterization of peripancreatic collections^[8-10]. Amano *et al*^[11] reported 12 patients with mild AP who had CT and MRI examinations, and found non-enhanced MR imaging was superior to CT for depiction and confirmation of mild AP. However, there was a variety of intermediary forms from mild to severe pancreatitis which occurred in clinical practice^[3]. We hypothesized that IEP may include mild AP and some intermediary forms based on clinical classification of AP, and that non-enhanced MR imaging could depict these abnormalities of IEP.

MATERIALS AND METHODS

Patients

All patients in this study were diagnosed as IEP based on clinical features and laboratory findings in our institute from January 2002 to March 2005. The recruitment criteria of patients were: (1) acute history; (2) pancreatitis at first onset; (3) elevated amylase or lipase, excluding other abnormalities with an elevated enzymes; (4) MRI examination; (5) maximum three-days interval between the MRI examination and the IEP onset; (6) minimal organ dysfunction or an uneventful recovery; and (7) no clinical or imaging appearance of pancreatic necrosis, pseudocyst, or abscess.

In this retrospective study, 53 patients (26 men and 27 women; average age: 47 ± 16 years, range: 10-73 years) with IEP were enrolled according to the aforementioned criteria. Medical records of the 53 patients were reviewed to investigate the recovery and the length of hospital stay. Review of images and medical records was conducted according to our Institutional Review Board's guidelines, including approval for our department to conduct these reviews.

MR Imaging

All examinations were conducted on a 1.5-T MR scanner with 38 mT/M gradients and 120 mT/M per second slope (Signa Excite; GE Medical Systems, Milwaukee, Wis), using phased-array torso-pelvis coil. The sequences included axial fast spoiled gradient echo (FSPGR) T1-weighted imaging with fat suppression, gradient-echo (GRE) T1-weighted in-phase and out-of-phase MR imaging, respiratory-triggered (R-T) axial fast recovery fast spin-echo (FRFSE) T2-weighted MR imaging with fat suppression, coronal and axial single shot fast spin-echo (SSFSE) T2-weighted MR imaging, and three-dimension (3D) FSPGR dynamic enhanced MR imaging.

FSPGR T1-weighted imaging with fat suppression was obtained in 1 or 2 breathholds, with the following parameters: TR ms/TE ms = 150-170/1.6; flip angle = 80°; matrix = 512×160 -192; field of view = 26-32 cm; section thickness = 5 mm (gap ≤ 0.5 mm); number of signals acquired (NSA) = 1; and the sampling bandwidth

= 20.8 kHz.

GRE in-phase and out-of-phase MR imaging were acquired in breath hold, with the following parameters: TR ms/TE ms = 150/4.4, 2.2; flip angle = 90°; matrix = 256×192 -224; field of view = 26-32 cm; section thickness = 5 mm (gap ≤ 0.5 mm); number of signals acquired (NSA) = 1; and the sampling bandwidth = 31 or 62 kHz.

R-T FRFSE T2-weighted sequence were obtained with the following parameters: repetition time [TR] ms/echo time [TE] ms = 10 000-12 000/90-100, TR determined by the frequency of respiration; section thickness = 5 mm; intersection gap = 0.5 mm; matrix = 256×192 ; number of signals acquired (NSA) = 3; and field of view = 34 cm \times 34 cm. It took about 3-4 min to complete the acquisition.

Coronal and axial SSFSE T2-weighted images were obtained in breathhold, with the following parameters: TE = 90-100 ms; 2 s between slice acquisitions; section thickness = 5 mm; intersection gap = 0.5 mm; matrix = 384×224 ; one-half signal acquired; and field of view = 33 cm \times 33 cm.

Radial oblique slab SSFSE images were obtained for MRCP with the following parameters: TE = 1300 ms; 6 s between image acquisitions; section thickness = 40 mm; matrix = 384×224 ; one-half signal acquired; and field of view = 30 cm \times 30 cm.

Dynamic enhanced imaging was performed with axial fat saturated 3D FSPGR sequence. Gadolinium chelate was administered (0.2 mmol/L per kilogram of body weight) intravenously at approximately 3.5 mL/s by projector (Spectris MR Injection System, Medrad Inc., USA) injection, followed by a 20-mL saline solution flush. An additional delayed phase was acquired using 2D FSPGR fat suppression axial T1-weighted imaging.

Image interpretation

The original MR imaging data was loaded onto a workstation (GE, AW4.1, Sun Microsystems, Palo Alto, CA) to be reviewed. Two observers (with 4 and 6 years' experience in interpreting abdominal MRI examinations), who were blinded to the laboratory data and clinical outcome, reviewed MR images. Any discrepancy between the two readers was settled by consensus.

The enlarged pancreas was defined as anterior-posterior diameter ≥ 3 cm on axial images^[12]. Decreased signal intensity of pancreas on FSPGR T1-weighted imaging was defined as not higher than that of liver^[13,14]. The signal intensity of normal pancreas was defined as slightly higher than that of liver on T2-weighted images^[13]. Pancreatic lobular septum thickening (PLST) with increased signal intensity was defined as a thickness of the septum surrounding pancreatic lobules ≥ 2 mm and its signal intensity significantly higher than that of liver. The pancreatic fascial plane (PFP) was defined as linear signal intensity along the surface of the pancreas which was hypointense on in-phase images or hyperintense on T2-weighted images.

Peripancreatic fat stranding (PPFS) referred to the stranding with hypointensity on in-phase images or hyperintensity on T2 weighted images. Peripancreatic fluid (PPF) collections referred to poorly defined fluid

collection in the anterior pararenal space around the gland and in the lesser peritoneal sac, with diameter larger than 2 cm. The renal fascial plane (RFP) was defined as renal fascia thickness over 3 mm^[15], which was hypointense on in-phase and hyperintense on T2-weighted images. Perivascular fluid referred to fluid surrounding portal vein, superior mesentery vein (SMV) or artery (SMA), which was hyperintense on T2-weighted images.

The diameter of the pancreatic main duct was measured at the level of the head or body of the pancreas on MRCP images. Pancreatic necrosis was also noted on gadolinium chelate 3D FSPGR dynamic enhanced images.

The grade of the pancreatitis was scored with the MR severity score index (MRSI) (Table 1), which was derived from the CT severity score index developed by Balthazar and others^[4,15-17]. The severity of pancreatitis was expressed as mild (score = 0-2 points), moderate (score = 3-6 points), and severe (score = 7-10 points).

Statistical analysis

Results were expressed as mean \pm SD for continuous data. Two-tailed student's *t* test was used to calculate differences between two observers. The Chi-square test was used to evaluate the difference for MR findings among IEP patients on in-phase, SSFSE T2- and R-T T2- weighted imaging, as well as to compare the prevalence of MR findings for IEP on R-T T2- weighted images. Relationship of length of hospitalization variables to the severity of IEP was tested using *t* tests, and to the continuous variables with Pearson product-moment correlations. *P* < 0.05 was considered statistically significant.

RESULTS

Sample characteristics

In this study, 49% (26/53) of patients had cholecystopathy or cholelithiasis confirmed by surgery or laparoscopy. One patient had congenital choledochocoele and cholecystitis, confirmed at laparotomy. Of them, 17% (9/53) patients had alcohol abuse, and 34% (18/53) were idiopathic.

MRI findings

The anterior-posterior diameter of the pancreas on FSPGR T1-weighted imaging was, respectively, 2.3 ± 0.4 cm and 2.4 ± 0.4 cm (*P* = 0.1) for observer 1 and observer 2. Only one patient had a diameter of the pancreas larger than 3 cm.

Of the 53 patients with IEP, 89% (47/53) had at least one abnormality on MR images, whereas 11% (6/53) showed normal pancreas. We found that 19% (10/53) of patients showed decreased pancreatic signal intensity on FSPGR T1-weighted images, while 81% (43/53) of patients showed normal pancreatic signal intensity. In addition, 25% (13/53) and 30% (16/53) of patients showed increased signal intensity on SSFSE T2-weighted and R-T T2-weighted images (Figure 1), respectively (χ^2 = 0.5-1.8, *P* > 0.05). As shown in Figures 1 and 2, 21% (11/53) and 40% (21/53) of patients had PLST with increased signal intensity on SSFSE T2-weighted and R-T T2-weighted images, respectively (χ^2 = 4.5, *P* < 0.05).

The other MR findings of IEP are shown in Table 2,

Table 1 MR severity score index

Prognostic indications	Characteristic	Points
Pancreatic	Normal pancreas	0
Inflammation	Focal or diffuse enlargement of the pancreas	1
	Intrinsic pancreatic abnormalities with inflammatory changes in pancreatic fat	2
	Single, poorly defined fluid collection or phlegmon	3
	Two or more poorly defined collection or presence of gas in or adjacent to the pancreas	4
Pancreatic necrosis	No necrosis	0
	30%	2
	30%-50%	4
	> 50%	6

and illustrated in Figure 1, Figure 2, Figure 3, Figure 4. The prevalence of most findings of IEP on R-T T2-weighted imaging was higher than that on in-phase or SSFSE T2-weighted imaging (Table 2). The commonest finding of IEP on R-T T2-weighted imaging was PFP (85%), left RFP (77%) and PPFS (55%), whose prevalence was significantly higher than those for PPF, right RFP, perivascular fluid and free ascites, respectively (Table 2) (χ^2 = 78.5, *P* < 0.001).

All of the 29 (54.71%) patients with PPFS and 13 (24.52%) patients with PPF had PFP. All these 13 patients had lesser peritoneal sac fluid collections, including 5 with collections in the left anterior pararenal space fluid collections, 1 with a right anterior pararenal space fluid collection, and 2 with both right and left anterior pararenal space fluid collections.

Of 53 patients, 43 (81%) showed RFP, including 22 (42%) with both left and right RFP, 1 (2%) with only right RFP and 20 (38%) with only left RFP. The number of patients with left RFP was significantly higher than those with right RFP (42 *vs* 23 cases; χ^2 = 14.4, *P* < 0.001).

On MRCP images, the main pancreatic duct was smooth, and its diameter was 2.2 ± 0.7 mm and 2.1 ± 0.6 mm (*P* = 0.7) for observer 1 and observer 2, respectively. No pancreatic necrosis was seen on gadolinium chelate 3D FSPGR dynamic MR imaging or other sequences.

Length of hospitalization and MRSI

The average length of hospitalization of the 53 patients was 14 ± 7 d (range: 4-33 d). The MRSI ranged from 0 points to 4 points with the average of 2.1 ± 1.2 points. The IEP was graded as mild in 37 (70%) patients, as moderate in 16 (30%) patients and as severe in 0 patients. The severity of pancreatitis was not related to the length of hospitalization (*r* = 0.1, *P* > 0.05).

DISCUSSION

In our study, we found 89% of patients with IEP had at least one abnormality on non-enhanced MR imaging, depicted primarily on T2-weighted images. These abnormalities mainly included pancreatic fascial plane (85%), left renal fascial plane (77%), peripancreatic fat

Table 2 MR findings of IEP in 53 patients

	PFP n (%)	PPF n (%)	PPFS n (%)	LRFP n (%)	RRFP n (%)	PVF n (%)	FA n (%)
In-phase	18 (34)	10 (19)	15 (28)	26 (49)	8 (15)	1 (2)	7 (13)
SSFSE T2W	33 (62.3)	13 (25)	24 (45)	31 (58)	16 (30)	14 (26)	10 (19)
R-T T2W	45 (85)	13 (25)	29 (55)	41 (77)	22 (42)	24 (45)	10 (19)
χ^2 (P values) ¹	28.9 (< 0.001)	0.6 (0.7)	7.8 (0.02)	9.3 (0.01)	9.1 (0.01)	27.1 (< 0.001)	0.8 (0.7)

PFP: Pancreatic fascial plane; PPF: Peripancreatic fluid; PPFS: Peripancreatic fat stranding; LRFP: Left renal fascial plane; RRFP: Right renal fascial plane; PVF: Perivascular fluid; FA: Free ascites. ¹Comparison among three sequences.

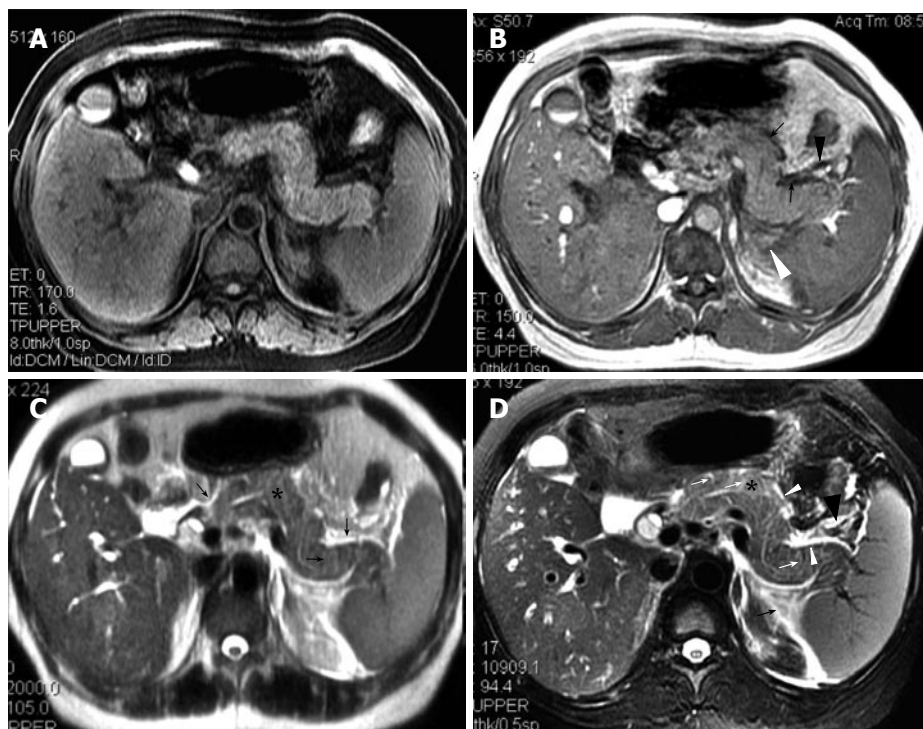


Figure 1 A 42-year-old woman with interstitial edematous pancreatitis. **A:** SPGR fat-suppressed T1-weighted (TR/TE = 170/1.6 ms) image shows pancreatic signal intensity comparable to that of liver; **B:** GRE in-phase (TR/TE = 150/4.4 ms) image shows pancreatic fascial plane (arrows) and peripancreatic fat stranding (arrow heads); **C:** SSFSE T2-weighted (TE = 90 ms) image shows increased pancreatic signal intensity (asterisk). Stranding in the pancreatic fascial plane (large arrow) and peripancreatic fat and thickened pancreatic lobular septum (small arrow) can also be seen; **D:** R-T T2-weighted (TR/TE = 11 800/93 ms) image shows above findings much better (Asterisk indicates pancreatic parenchyma, white arrows indicate thickened pancreatic lobular septum, white arrow head indicates pancreatic fascial plane, and black arrow and arrow head indicate fat stranding).

stranding (55%), peripancreatic fluid (55%), perivascular fluid (45%), right renal fascial plane (42%), and pancreatic lobular septum thickening (40%). R-T T2-weighted imaging was much more sensitive than in-phase and SSFSE T2-weighted imaging in the depiction of the aforementioned abnormalities.

The pathology of IEP included pancreatic edema and mild cellular infiltration, sometimes with a few small scattered foci of necrosis and saponification in the peripancreatic fatty tissue^[3]. The histological changes noted in experimental pancreatitis are progressive interstitial edema, and mononuclear-cell infiltration, without hemorrhage or necrosis, with regeneration of the pancreas in 6 d^[18]. In our study, 19% of patients showed decreased pancreatic signal intensity on FSPGR T1-weighted images, while 25%-30% of patients showed increased pancreatic signal intensity on SSFSE T2-weighted and R-T T2-weighted images. The abnormal signal intensity of pancreatic parenchyma may partly reflect the inflammation of the pancreas in IEP. However, our results indicate that the signal intensity of the pancreatic parenchyma on both T1-weighted and T2-weighted imaging is not sensitive for depicting IEP. In the 12 patients with mild acute pancreatitis reported by Amano *et al*^[11], 75% (9/12)

of patients showed a prolonged T1 and T2 lesions of the pancreas. However, the patients selected in our study differ from those in theirs. IEP is not the same as mild acute pancreatitis, including a variety of intermediary forms from mild to severe pancreatitis which occur in clinical practice^[3].

The pancreas does not have a well-developed fibrous capsule, but is surrounded by thin loose connective tissue, which is called the “fusion fascia of Treita” in the head and the “fusion fascia of Toldt” in the body and tail of the pancreas^[19]. In our study, we found the commonest sign was PFP on T2-weighted images (85%). In the 29 patients with PPFS and 13 patients with PPF, all had PFP. We speculate that the pancreatic fascia may be first involved in acute pancreatitis. When the inflammation penetrated through the Toldt fusion fascia of the pancreas, peripancreas fat infiltration or fluid accumulation may develop. The peripancreatic vessels run in the fusion fascia^[19]. It is possible that peripancreatic perivascular fluid in IEP resulted from inflammation of intra-fusion fascia of the pancreas.

In AP, the earliest changes appear to be in the acinar cells^[20]. An increase in pancreatic ductal pressure has been described as the initial mechanism of acinar cell injury. The

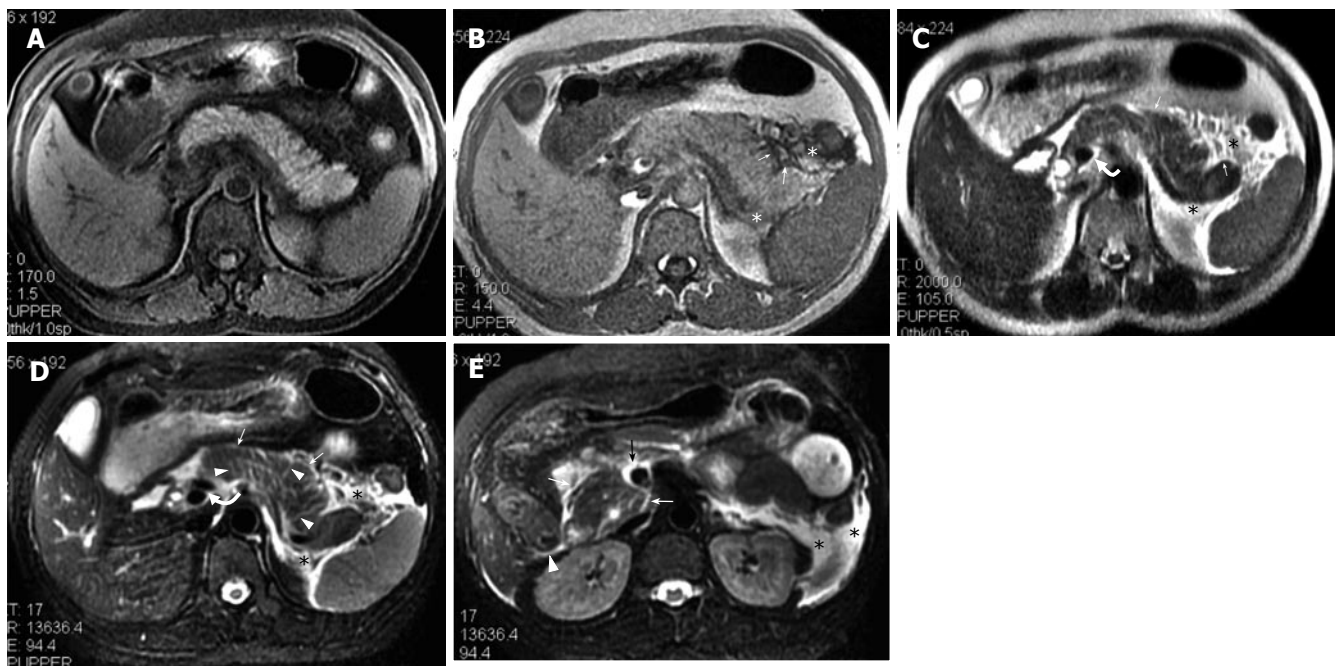


Figure 2 A 28-year-old man with IEP. **A:** SPGR fat-suppressed T1-weighted (TR/TE = 170/1.6 ms) image shows normal pancreatic signal intensity; **B:** pancreatic fascial (arrows) and peripancreatic fat (asterisk) stranding can be seen on GRE in-phase (TR/TE, 150/4.4 ms) image; **C:** SSFSE T2-weighted (TE = 90 ms) image; **D:** R-T T2-weighted (TR/TE = 12300/98 ms) image. Perivascular fluid (curve arrow) can be seen on latter two sequences. Thickened pancreatic lobular septum (arrow heads) can only be seen on R-T T2-weighted image (**D**); **E:** At the level of the head of the pancreas, R-T T2-weighted image (**E**) shows pancreatic fascial plane (white arrow), right renal fascia plane (white arrow head), fluid surrounding SMV (black arrow), and left anterior pararenal space and lateroconal plane fluid collections (asterisk).

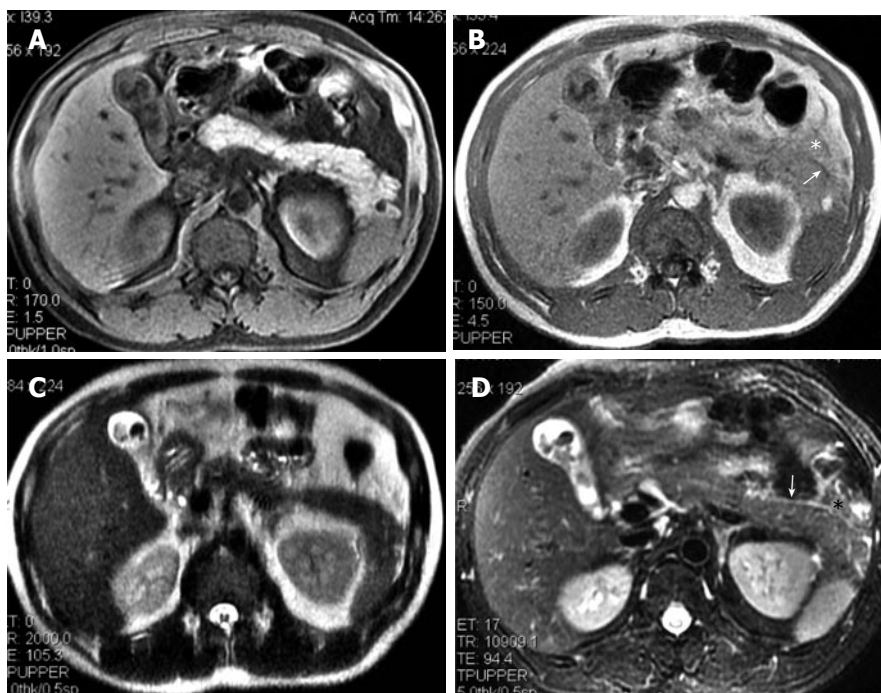


Figure 3 A 45-year-old woman with IEP and cholelithiasis. **(A)** SPGR fat-suppressed T1-weighted (TR/TE = 170/1.6 ms) image shows normal pancreatic signal intensity. GRE in-phase (TR/TE = 150/4.4 ms) **(B)** and SSFSE T2 weighted (TE = 90 ms); **(C)** images show the pancreatic fascia plane (arrows) and the peripancreatic fat stranding (asterisk), which appear more extensive on R-T T2-weighted (TR/TE = 12 300/98 ms) image (**D**).

subsequent cellular events are not well understood, but the result appears to be activation of pancreatic digestive enzymes within the acinar cell^[21]. Ultrastructural studies by Kloppel *et al*^[22] indicate that an early event, irrespective of etiology, is the release of activated enzymes by acini at the periphery of lobules into the interstitium, resulting in perilobular fat necrosis. Experimentally, tracer material introduced by retrograde injection into the main pancreatic duct (MPD) is localized to the periacinar space^[23] and

human AP may show a similar pattern, irrespective of etiology^[24]. Early microcirculatory changes in patients with AP include an increase in vascular permeability and the accumulation of extravagated fluid in the perilobular space. A decrease in flow velocity was noted 2 h after the onset of severe pancreatitis^[25]. We found that 21%-40% of patients had PLST with increased signal intensity on T2-weighted images. We speculate that this resulted primarily from the accumulation of extravagated fluid in the

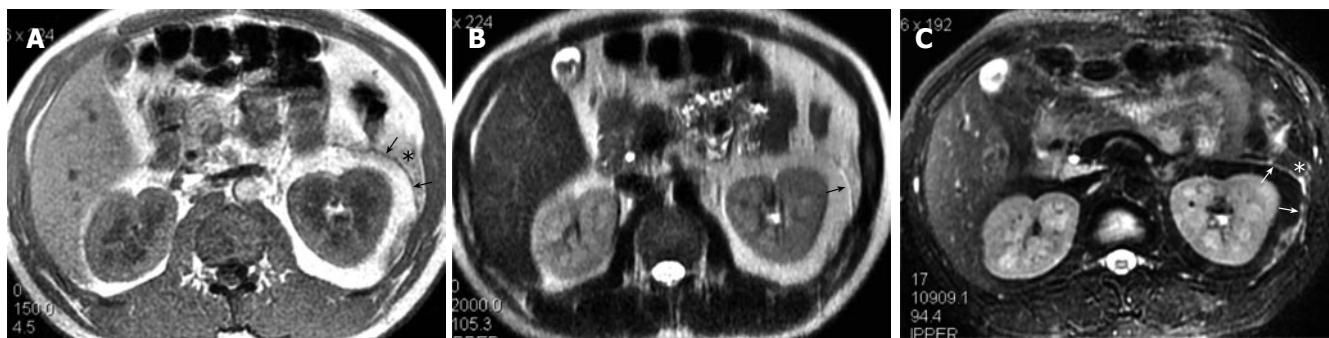


Figure 4 The same patient as in Figure 3. At the level of the head of the pancreas, left renal fascial plane (arrow) and anterior pararenal space fat stranding (asterisk) present on GRE in-phase (A), SSFSE T2-weighted (B) and R-T T2-weighted (C) images, but they are most prominent on R-T T2-weighted image.

perilobular space and perilobular fat necrosis.

CT has been helpful in revealing inflammatory thickening of the retroperitoneal fascial membranes and edema or lipolysis of the retroperitoneal fat resulting from acute pancreatitis. Nicholson *et al*^[15] reported that 46% of patients with pancreatitis demonstrated renal fascial thickening and 27% of patients had increased fat density. Chintapalli *et al*^[16] reported that renal fascial thickening was seen in 62% of patients with pancreatitis, involving left and right renal fascia in 59% (42/71) and 30% (21/71), respectively. The patients in the above two reports had various forms of pancreatitis. In our study, 81% of patients with IEP showed RFP, including 77% with left RFP and 42% with right RFP. The prevalence of left RFP was significantly higher than that of right RFP ($P < 0.05$), although the prevalence of RFP in MRI was higher than that reported in CT^[15,16]. It is possible that MR imaging is more sensitive than CT for detecting renal fascia inflammatory thickening. The predominance of left RFP is consistent with earlier studies by CT, attributed to the proximity of the body and tail of the pancreas^[16].

Mortelet *et al*^[26] reported that the pancreas appeared abnormal on MRI and MRCP in 57% (31/54) patients with asymptomatic mild serum hyperamylasemia and hyperlipasemia, while 43% of patients had normal pancreas. In our study, 11% (6/53) of patients showed normal pancreas on MR imaging. Each of these 6 patients had 3 times or more elevations of serum amylase and lipase at hospital admission, and serum amylase and lipase levels decreased within a few days. It is possible that the pancreatitis in these 6 patients was too mild to be seen with current non-enhanced MR imaging.

In our study, we did not find any correlation between the length of hospitalization and MRSI ($r = 0.1$, $P > 0.05$), which was in agreement with the findings by Lecesne *et al*^[8]. Most of the patients with IEP were graded as mild (70%), and none were graded as severe in our study. Also, 49% of patients had cholecystopathy or cholelithiasis. We agree with Lecesne *et al*^[8] that the length of hospitalization may not accurately represent morbidity, since social factors or selective surgery may prolong the length of stay, which was not included in the design of our study.

We also did not analyze the correlation of the level of serum amylase and lipase to the MR findings or to the length of hospitalization. Reports in the early literature

indicated that the severity of acute pancreatitis was independent of the elevation in serum amylase/lipase level (≤ 3 or > 3 times of higher limit of normal range) on admission. Patients with only a slight increase can also have or develop severe acute pancreatitis^[27]. In diabetic ketoacidosis, non-specific elevations of amylase and lipase occurred in 16%-25% of cases. Diagnosis of acute pancreatitis based solely on elevated amylase or lipase, even > 3 times normal, is not justifiable^[28]. Mortelet *et al*^[26] reported that 43% of patients with asymptomatic serum hyperamylasemia and hyperlipasemia had normal pancreas on MRCP. The above reports indicate that the level of serum amylase and lipase was not relative to the findings on MR imaging and the severity of acute pancreatitis.

In our study only 3 patients had repeated MR imaging, so we were not able to document serial changes in the development of pancreatic inflammation. However, the short-term clinic status of all patients improved. Munoz-Bongrand *et al*^[29] reported that serial computed tomography is rarely necessary in patients with acute pancreatitis, and repeated MR examinations may similarly be unnecessary in patients with IEP.

In conclusion, interstitial edematous pancreatitis primarily manifests on unenhanced MR images as the pancreatic fascial plane, left renal fascial plane and peripancreatic fat stranding, as well as peripancreatic fluid. Non-enhanced MR imaging, especially R-T T2-weighted imaging with fat suppression, is effective for depicting interstitial edematous pancreatitis.

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RAPID COMMUNICATION

The epidemiology of hyperferritinaemia

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Abstract

AIM: To discover the causes of markedly raised ferritin levels in patients seen at a teaching hospital in Newcastle Upon Tyne, United Kingdom.

METHODS: Demographic and medical data were collected for all patients over 18 years who had a serum ferritin levels recorded as $\geq 1500 \mu\text{g/L}$ during the period January to September 2002. The cause or causes for their hyperferritinaemia were identified from their medical notes. Patients from a defined local population were identified.

RESULTS: A total of 19 583 measurements were provided of which 406 from 199 patients were $\geq 1500 \mu\text{g/L}$. An annual incidence for the local population was determined to be 0.44/1000. 150/199 medical notes were scrutinised and 81 patients were identified as having a single cause for their raised ferritin level. The most common single cause was alcoholic liver disease in the local population and renal failure was the most common single cause in the overall population. Confirmed hereditary haemochromatosis was the 10th most common cause. Liver disease contributed to hyperferritinaemia in 44% of the patients. Weight loss may have contributed to hyperferritinaemia in up to 11%.

CONCLUSION: Alcohol related liver disease, haematological disease, renal failure and neoplasia are much more common causes of marked hyperferritinaemia than haemochromatosis. The role of weight loss in hyperferritinaemia may warrant further investigation.

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Key words: Ferritin; Epidemiology; Haemochromatosis

INTRODUCTION

Ferritin is a protein with a molecular mass of 450 kilo Daltons (kD) and is composed of 24 subunits^[1]. These subunits form a hollow sphere, which can contain up to 4000 atoms of iron stored as ferric oxyhydroxide phosphate; haemosiderin is a condensate of ferritin. Ferritin acts as an intracellular store of iron whilst the protein coat protects the cell from potentially toxic ionised iron. In normal and iron overload states, serum ferritin levels correlate with mobilisable, stored iron. Iron overload may result from disease; e.g. haemochromatosis, administered iron or iatrogenically from recurrent blood.

Raised ferritin levels that are not due to iron overload are seen in many other conditions. Parenchymal liver damage, infection, inflammation (e.g. rheumatoid arthritis) and malignant disease may all be associated with raised serum ferritin. It has also been postulated that weight loss per se may be associated with elevated levels of ferritin^[2].

Clinicians may request a serum ferritin test to exclude iron deficiency in an anaemic patient or when considering a condition that is associated with elevated levels of ferritin. However, clinicians may not be aware of all the reasons for markedly elevated levels of ferritin and the relative frequencies of these different causes, and therefore may miss a potentially important diagnosis.

We conducted a study to look at the frequency of various diseases in a patient group with markedly raised ferritin levels who lived within a defined area. The patient group was made up of those seen at a teaching hospital that had various specialist units. We also looked at weight loss as a contributing factor in hyperferritinaemia.

MATERIALS AND METHODS

Patients

The Newcastle Hospital's NHS Trust serves a local population of around 270 000 and is a tertiary referral centre for up to 4 million people. Among other services it provides the regional renal transplant, liver and haematology service.

All patients aged over 18 years who had serum ferritin

Table 1 Conditions associated with raised ferritin and diagnostic criteria used

Condition	Requirements for diagnosis
Hereditary haemochromatosis	Genotyped and / or biopsy proven
Renal failure	Dialysis dependent
Alcoholic liver disease (ALD)	Liver biopsy or Liver Function Test (LFT) abnormality compatible with ALD in patients with history of alcohol excess (> 30 u/wk) in whom other causes have been excluded
Inflammatory disease	Raised C-Reactive Protein (CRP) and/or Erythrocyte Sedimentation Rate (ESR) on more than one consecutive test and recognised active inflammatory disease
Repeated blood transfusion	More than 4 unit packed cell transfusion in preceding 6 mo
Autoimmune disease	Recognised autoimmune disease with positive auto-antibody test
Other liver disease	All other recognised causes of parenchymal liver damage with abnormal LFTs, excluding ALD and HHC
Haematological disease	Bone marrow or blood film proven primary haematological disorder
Neoplasia	Histologically proven neoplastic disease
Weight loss	More than 10% body mass (kg) lost in preceding six months
Human immuno-deficiency virus (HIV)	Positive HIV test

Table 2 Causes of ferritin ≥ 1500 $\mu\text{g/L}$ in local and general population

Condition	All patients, $n = 150$ (% total)	Local patients, $n = 71$ (% total)	Number with condition as single cause for raised ferritin (%)
HHC	13 (8.6)	8 (11.3)	5/13 (38.5)
Alcoholic liver disease	33 (22)	22 (31.0)	24/33 (72.7)
Other liver disease	20 (13.3)	10 (14.1)	8/20 (40.0)
Inflammatory Disease	28 (18.6)	9 (12.6)	7/28 (25.0)
Neoplasia	29 (19.3)	11 (15.4)	6/29 (20.7)
Repeated blood transfusion	26 (17.3)	9 (12.6)	1/26 (3.8)
Autoimmune disease	21 (14.0)	8 (11.3)	2/21 (9.5)
Haematological disease	38 (25.3)	11 (15.4)	6/38 (15.7)
Renal failure	42 (28.0)	15 (21.1)	20/42 (47.6)
Weight loss	17 (11.3)	6 (8.4)	2/17 (11.7)
HIV	1 (0.7)	1 (1.4)	0/1 (0)
Unexplained	3 (2.0)	3 (4.2)	3/3 (100)

assayed between January 2002 and September 2002 were identified. All those with a ferritin level of ≥ 1500 micrograms/litre ($\mu\text{g/L}$) (normal range 12–200 $\mu\text{g/L}$) were included in the study. Demographic data, medical histories (including blood transfusion history and documented weight loss), biochemical markers and histopathological data were collected for each patient. The medical notes of patients with specific diagnoses, such as Hereditary Haemochromatosis (HHC), were scrutinised to ensure diagnostic criteria had been met before the patients were classified^[3] (Table 1).

RESULTS

19 583 ferritin level results were obtained for a nine month period (equivalent to 80 tests per day). 199 patients had serum ferritin results of ≥ 1500 $\mu\text{g/L}$ (from 406 samples among them). Hyperferritinaemia was thereby identified with an annual incidence of 0.44/1000 in the local population.

150/199 (75%) case notes were retrieved for 97 male

Table 3 The most common causes of hyperferritinaemia

All patients (%)	Local Patients (%)
Renal failure (28.0)	Alcoholic liver disease (31.0)
Haematological disease (25.3)	Renal failure (21.1)
Alcoholic liver disease (22)	Neoplasia (15.4)
Neoplasia (19.3)	Haematological disease (15.4)
Inflammatory disease (18.6)	Other liver disease (14.1)
Repeated blood transfusion (17.3)	Repeated blood transfusion (12.6)
Autoimmune disease (14.0)	Inflammatory disease (12.6)
Other liver disease (13.3)	HHC (11.4)
Weight loss (11.3)	Autoimmune disease (11.4)
HHC (8.6)	Weight loss (8.4)
Unexplained (2.0)	Unexplained (4.2)
HIV (0.7)	HIV (1.4)

patients and 53 female patients (ratio 1.8:1). The median age (taken at 1/1/02) was 54 years. 71 (47.3%) were “local” from Newcastle upon Tyne (postcode NE1 - NE12) (Table 2). Of the notes we were unable to obtain, 27/49 were local patients (55%) and 30/49 were male (ratio 1.6:1). The median age of this group was 54 years.

Causes of raised ferritin in general and local populations

Of the 150 patients evaluated in detail, 81 had one single identifiable cause for their hyperferritinaemia, with only 3 patients having none of the listed potential causes (Table 1) recorded in their medical records as well as no significant weight loss. Table 2 gives details of the frequency of conditions associated with a markedly elevated serum ferritin level. Table 3 lists the 10 most common causes for hyperferritinaemia in all patients studied as well as for patients who lived locally.

Renal failure was the most common cause for hyperferritinaemia in the overall patient population of the hospital trust that provides significant tertiary care, however, alcoholic liver disease was the most common cause of a raised ferritin level in the local population. 51% of patients in our study had only one cause identified for their hyperferritinaemia, with alcoholic liver disease being the most common single cause for a raised ferritin

level. Hereditary haemochromatosis accounted for only 9% of cases, ranking as the 10th commonest cause of hyperferritinaemia. Liver disease (alone) accounted for 25% of cases, but contributed to raised ferritin levels in 44%.

Degree of hyperferritinaemia

The median ferritin level was 2613 µg/L. Those with liver diseases had the highest ferritin levels (Table 4). Patients with haematological disease (or repeated blood transfusion) as the single cause of a ferritin level ≥ 1500 µg/L also had very high median ferritin level results. Patients with renal failure had relatively lower median ferritin level results.

DISCUSSION

Tests to determine serum ferritin levels are frequently requested, and causes of a markedly raised ferritin level are multiple. This study concentrated on the conditions found in patients known to have hyperferritinaemia, and did not look at the initial indication for checking the serum ferritin level. Clinicians may not routinely check serum ferritin in all patients with, for example, normal liver function tests or normal haemoglobin, and thus our list of associated conditions may not accurately reflect all the causes of hyperferritinaemia.

We chose 1500 µg/L (more than seven times the upper limit of normal) as our cut-off for defining hyperferritinaemia. This gave us a large enough study cohort to provide meaningful data, whilst producing a manageable number from which to accurately retrieve patient information.

Hereditary Haemochromatosis

Dying red blood cells in macrophages are broken down to release iron to serum transferrin, which is then taken up by transferrin receptors on parenchymal tissues and in the bone marrow. In HHC, it is thought the binding of transferrin receptor 1 is inhibited in crypt and reticuloendothelial cells via the protein hepcidin^[4]. This leads to raised ferritin, high transferrin saturation and high liver iron concentration.

HHC is a relatively rare cause of markedly raised ferritin ranking as the 8th most common cause in the local population. Local guidelines for the diagnosis of HHC suggest that unless the transferrin saturation is $> 45\%$ genotyping is not indicated^[3]. A serum ferritin greater than 300 µg/L (for men and post-menopausal women) is regarded as requiring further investigation for HHC. Our cut-off of 1500 µg/L is likely to have missed a large proportion of patients with HHC, which would include both those with a mild degree of iron overload and patients with HHC undergoing successful venesection treatment. HHC has a prevalence of 1/300 (which should be 900 patients locally). Clearly, if stable patients are having their ferritin levels checked twice yearly as recommended, our study would have missed a large proportion of these patients, suggesting that local venesection therapy is working.

It is well-known now that patients who are ho-

Table 4 Median ferritin levels in each condition

Condition (n)	Median ferritin µg/L	Number with condition as single identifiable cause for ferritin ≥ 1500 µg/L	Median ferritin in those with = cause for ferritin ≥ 1500 µg/L
HHC (13)	5031	5	7432
Other liver disease (19)	2889	8	3055
Unexplained (3)	2606	n/a	n/a
Weight loss (16)	2508	2	2160
ALD (31)	2484	24	2121
Autoimmune disease (21)	2203	2	2017
Haematological disease (31)	2075	5	3974
Inflammatory disease (24)	1995	3	2877
Repeated blood transfusion (29)	1977	1	6315
Renal failure (40)	1975	20	1954
Neoplasia (26)	1767	3	2409
HIV (1)	1711	0	n/a

mozygous for HHC and who drink more than 60 g alcohol per day are approximately 9 times more likely to develop cirrhosis than those who drink less. This is associated with a massive rise in iron concentrations on biopsy^[5]. All eight patients with HHC who had more than one identifiable risk factor for hyperferritinaemia were heavy drinkers.

Other Liver Diseases

The liver diseases (particularly HHC) produce the most markedly raised ferritin levels, especially in those with no other contributing factors (Table 4). There is evidence now of an association between iron excess on liver biopsy with other metabolic disorders^[6]. A study in 2001 confirmed that an increased ferritin level with normal transferrin saturation is frequently found in patients with hepatic steatosis^[7] but reflects iron overload only in those in whom it persists despite an appropriate diet. We did not include non-alcoholic steatohepatitis or fatty liver disease as separate diagnoses, nor did we look at associated metabolic disease, other than HHC, but it seems likely given the above evidence, that some of the patients in the “other liver disease” category had these conditions.

Investigating patients who were referred with abnormal liver function tests who were found to have a raised serum ferritin level is potentially time-consuming and costly. Time and resources could be reduced if cut-off levels for serum ferritin, above which specific diagnoses could be included or excluded from the differential diagnosis, were available. To do this most effectively, we would need to look at median ferritin levels at diagnosis for all patients with each of the liver diseases (e.g., ALD, HHC, fatty liver, autoimmune hepatitis) and not just at those patients identified with marked hyperferritinaemia. This may then enable greater targeting in subsequent investigations. From our limited study, it seems likely that patients with massively raised ferritin levels (e.g. > 5000 µg/L) are more likely to have HHC than any of the other chronic liver diseases or conditions commonly associated with hyperferritinaemia.

Weight Loss

More patients had weight loss as an identifiable potential cause of their hyperferritinaemia than had haemochromatosis. The weight loss in the majority of these patients was associated with neoplasia or chronic inflammatory disease. It is not possible to determine whether this was a significant factor in the development of hyperferritinaemia. Only two patients seemed to have weight loss alone as a cause for their hyperferritinaemia.

Patients with anorexia nervosa may be found to have increased ferritin and abnormal liver biopsies with high iron content. A study looking at malnourished children demonstrated abundant iron stores in the liver despite evidence of iron deficiency [personal communication, Professor Alan Jackson, University of Southampton]. The causes for this are unclear but it is postulated that the demand for oxygen carriage is reduced in the face of a diminished lean body mass, and hence red cell mass is lower due to the release of iron to be stored. It is also thought that bone marrow activity may be directly or indirectly suppressed due to ongoing stress or infection-related challenge, thus reducing demand on stored iron. Limited nutrient availability may also suppress marrow activity^[8]. These possible explanations are not mutually exclusive.

Renal Failure

Usual tests to diagnose iron deficient anaemia and measure iron stores are suspect in those with chronic renal failure, who are plagued by poor nutrition, multiple medications and acute and chronic inflammatory processes^[9]. Iron replacement therapy in chronic renal failure is common and these patients frequently undergo ferritin checks as a measure of their iron stores. There may be therefore a disproportionately high number of patients with chronic renal failure included in our study. The median ferritin level in patients with renal failure was 1959 µg/L (1954 µg/L in those with RF as a single cause). 52% (22/42) of patients

with renal failure had a ferritin level < 2000 µg/L making up 38% of all those with ferritin levels < 2000 µg/L in this study.

CONCLUSIONS

Serum ferritin level tests are frequently requested by clinicians. Clinicians should remember that alcohol related liver disease, haematological disease, renal failure and neoplasia are much more common causes of marked hyperferritinaemia than haemochromatosis. Further investigations into the role of weight loss as a contributing factor to raised ferritin levels are needed.

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RAPID COMMUNICATION

Nucleoporin 88 expression in hepatitis B and C virus-related liver diseases

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Abstract

AIM: To investigate the expression of nucleoporin 88 (Nup88) in hepatitis B virus (HBV) and C virus (HCV)-related liver diseases.

METHODS: We generated a new monoclonal Nup88 antibody to investigate the Nup88 protein expression by immunohistochemistry (IHC) in 294 paraffin-embedded liver specimens comprising all stages of hepatocellular carcinogenesis. In addition, in cell culture experiments HBV-positive (HepG2.2.15 and HB611) and HBV-negative (HepG2) hepatoma cell lines were tested for the Nup88 expression by Western-immunoblotting to test data obtained by IHC.

RESULTS: Specific Nup88 expression was found in chronic HCV hepatitis and unspecific chronic hepatitis, whereas no or very weak Nup88 expression was detected in normal liver. The Nup88 expression was markedly reduced or missing in mild chronic HBV infection and inversely correlated with HBcAg expression. Irrespective of the HBV- or HCV-status, increasing Nup88 expression was observed in cirrhosis and dysplastic nodules, and Nup88 was highly expressed in hepatocellular carcinomas. The intensity of Nup88 expression significantly increased during carcinogenesis ($P < 0.0001$) and correlated with dedifferentiation ($P < 0.0001$). Interestingly, Nup88 protein expression was significantly

downregulated in HBV-positive HepG2.2.15 ($P < 0.002$) and HB611 ($P < 0.001$) cell lines as compared to HBV-negative HepG2 cells.

CONCLUSION: Based on our immunohistochemical data, HBV and HCV are unlikely to influence the expression of Nup88 in cirrhotic and neoplastic liver tissue, but point to an interaction of HBV with the nuclear pore in chronic hepatitis. The expression of Nup88 in nonneoplastic liver tissue might reflect enhanced metabolic activity of the liver tissue. Our data strongly indicate a dichotomous role for Nup88 in non-neoplastic and neoplastic conditions of the liver.

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Key words: Nucleoporin 88; Hepatitis B and C virus; Hepatocellular carcinogenesis

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INTRODUCTION

Hepatocellular carcinoma (HCC) ranks fifth of the most common cancers worldwide and its incidence is rising in the Western world^[1]. Due to the high mortality associated with HCC it is the third leading cause of cancer death worldwide^[1]. The majority of HCCs develop due to chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection. However, the precise molecular etiology of HBV- and HCV- related HCC development remains poorly understood.

Bidirectional nuclear-cytoplasmic transport activity of oncogenes and tumorsuppressors through the nuclear pore is profoundly disturbed in cancer cells^[2]. It is assumed that transport through the nuclear pore is necessarily a crucial event in the development and maintenance of the tumor phenotype^[3]. Nucleoporin 88 (Nup88) is a protein located at the nuclear membrane and is involved in the bidirectional nuclear-cytoplasmic transport of proteins by forming nuclear pore complexes (NPC)

with other nucleoporins^[4,5]. Nup88 is associated in a dynamic subcomplex with CAN/Nup214, which has been implicated in nuclear protein import, nuclear mRNA export, and cell cycle regulation^[6]. Recently, overexpression of Nup88 has been demonstrated in a wide range of premalignant lesions and malignant tumors by using a polyclonal antibody^[7-9]. Thus, Nup88 has been suggested as a putative marker of malignant transformation. In addition, it has been shown that overexpression of Nup88 is involved in the tumorigenesis and aggressiveness of colorectal cancers^[10]. Overexpression of Nup88 is also linked to the enhanced metastatic potential of melanoma cells and it has been shown that Nup88 expression is up-regulated in primary foreskin melanocytes upon UV-A irradiation^[11,12]. In the present study we generated a new monoclonal Nup88 antibody and analyzed the expression of Nup88 in HBV- and HCV- related diseases of hepatocellular carcinogenesis in a large patient cohort and correlated major findings to cell culture experiments.

MATERIALS AND METHODS

Tissue samples

A total of 294 tissues were investigated for Nup88 expression by immunohistochemistry (IHC). One hundred and forty-one specimens were assessed on a liver tissue microarray (TMA) consisting of premalignant dysplastic nodules (DNs: $n = 33$) and hepatocellular carcinomas (HCCs: $n = 108$). Twenty-eight DN and 43 HCCs were infected with HCV, 16 DN and 17 HCCs were infected with HBV, and 11 DN and 8 HCCs were coinfecting with HBV and HCV. In addition, liver biopsies ($n = 153$) collected at the Institute of Pathology at Cologne were tested for Nup88 expression. These included cases of chronic HBV hepatitis ($n = 24$) and chronic HCV hepatitis ($n = 38$) with mild inflammatory activity, 53 cases of liver cirrhosis (HBV-positive, $n = 24$; HCV-positive, $n = 24$; alcohol induced, $n = 5$) and 18 cases of normal liver biopsies and 20 cases of mild unspecific chronic hepatitis (UCH), i.e. patients who underwent liver biopsies in the context of clinically elevated transaminases in the absence of HBV/HCV or any other known liver diseases.

Antibody production

Mouse monoclonal antibodies (mAbs) to protein Nup88 were obtained by immunization with the peptide representing the amino acid sequence (aa 1-263) deduced from the human cDNA sequence of Nup88 (NCBI, accession number: NP002523). As an immunogen the peptide was used to conjugate to keyhole limpet hemocyanin (KLH). For screening the carrier protein conjugated to the peptide was bovine serum albumin (BSA). Screening was performed using ELISA and Western immunoblotting following standard protocols. The mAbs were raised according to the method of Köhler and Milstein^[13].

Immunohistochemistry

Nup88 IHC was established on paraffin-embedded and formalin-fixed tissues. Colon adenomas and carcinomas ($n = 3$), breast carcinomas ($n = 2$) and lymph nodes with

Hodgkin's disease ($n = 3$) were used as positive controls^[8,9]. After deparaffinization and dehydration endogenous peroxidase was blocked by 1% H₂O₂ in methanol for 30 min. Without further pretreatment the slides were incubated with the mAb (clone 7, 1:750) in diluent buffer (Zymed Laboratories Inc., San Francisco, USA) at 4°C overnight. The Envision System HRP mouse (DakoCytomation, Carpinteria, USA) was applied to signal amplification for 30 min. For signal detection 3,3'-diaminobenzidine (DAB; Sigma Chemical Co., St. Louis, USA) was used. Slides were weakly counterstained with hematoxylin. Staining intensity and nuclear accumulation of Nup88 were evaluated by four independent observers and scored as: 0 = no signal; 1 = weak, pale nuclear staining; 2 = moderate staining; and 3 = strong staining. The HBV- and HCV-status was assessed by serology. Biopsies without serological information were tested for HBcAg and surface antigen (HBsAg) by IHC (see below) or by HCV-specific RT-PCR^[14]. Immunohistochemical double staining for HBcAg and Nup88 was performed on tissue specimens from mild chronic HBV hepatitis patients. In brief, the specimens were first treated with anti-HbcAg rabbit serum (DAKO, Hamburg, Germany) at a dilution of 1:400 for one hour at 37°C. After treatment with the secondary antibody for 30 min at room temperature, fast red alkaline phosphatase (Dako) was used as a chromogen. The Nup88 IHC was carried out as a second step according to the above protocol.

Cell culture experiments and Western immunoblotting

To analyze Nup88 expression in relation to HBV in cell culture, Nup88 and HbcAg expressions were tested in a hepatoma cell line. The HBV-negative HepG2 hepatoma cell line and its HBV-positive counterpart HepG2.2.15 were used^[15]. The latter contains the full length HBV genome and has been shown to produce infectious virions^[15]. Western immunoblots were performed as previously described using 75 g/L polyacrylamid gel^[17]. The monoclonal Nup88 antibody (clone 7, 1:2000 dilution) detected the 88 kDa Nup88 protein, the monoclonal HBcAg antibody (1:1000 dilution) and the 23 kDa HBcAg protein. Equal amounts of protein were loaded on the gel as tested by Bradford assay, Ponceau staining and GAPDH-Western blotting (Sigma, Germany; 1:1000) (Figure 3B).

Statistical analysis

For statistical analysis of the immunohistochemistry data of the TMA, chi-square test was applied. Densitometric evaluation of Western blot results from 6 independent experiments using *EASY plus Rev 4.16* were expressed as mean \pm SE. Results were compared using Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Nup88 expression in chronic HBV- and HCV-related hepatitis

In order to determine the Nup88 expression in viral hepatocellular carcinogenesis by IHC we first evaluated the efficacy of the new mAb Nup88 using different positive controls, i.e. colon carcinomas, breast carcinomas and

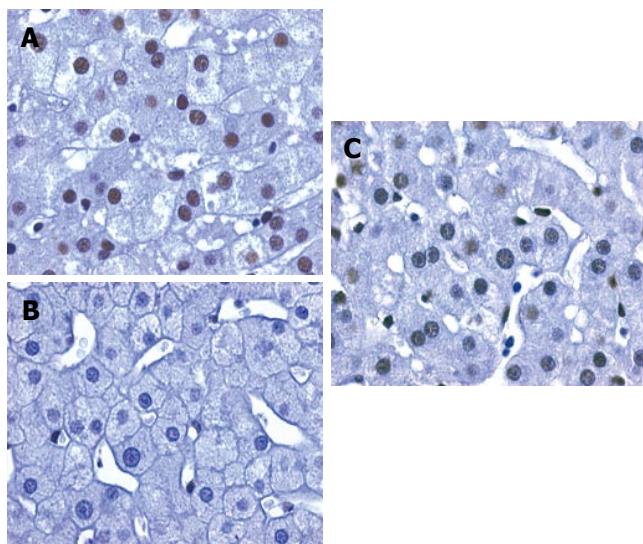


Figure 1 Nuclear Nup88 expression (brown nuclei) in non-neoplastic hepatocytes of a liver biopsy with mild unspecific chronic hepatitis (A), weak nuclear expression of Nup88 in chronic HBV-related hepatitis with mild inflammatory changes (B), and moderate nuclear expression of Nup88 in chronic HCV-related hepatitis with mild inflammatory changes (C).

lymph nodes with Hodgkin's disease^[8,9]. All positive controls demonstrated specific nuclear staining for Nup88 in the tumor cells whereas adjacent non-neoplastic tissue revealed no Nup88 expression. Of interest, no or only very weak Nup88 expression was detected in the 18 cases of normal liver biopsies, but in all 20 cases with mild reactive changes (mean staining score 2.4) (Figure 1A). In these cases Nup88 expression was found in most hepatocytes and occasionally in sinusoidal non-parenchymal liver cells. In contrast, in mild chronic HBV infection Nup88 expression was patchy, remarkably weaker or missing ($n = 24$, mean staining score 1.25) as compared to normal liver biopsies (Figure 1B). Stage-matched mild chronic HCV-infected liver samples ($n = 38$) revealed a moderate nuclear Nup88 expression (mean staining score 1.94; Figure 1C). No difference was observed in Nup88 expression between HBV- and HCV-related cirrhosis (mean staining score HBV-cirrhosis 1.91; HCV-cirrhosis: 2.04).

Double staining of Nup88 and HBcAg in mild chronic HBV-related hepatitis

In chronic HBV-related hepatitis with mild inflammatory activity HBcAg expression was patchy and restricted to single clusters of hepatocytes. To determine whether Nup88 was co-expressed with HBcAg in mild chronic HBV-related hepatitis we analyzed the relation between Nup88 and HBcAg expressions in mild chronic HBV-related hepatitis ($n = 5$) by IHC on serial sections and by IHC double staining. IHC staining for Nup88 and HBcAg on serial sections revealed a mutually exclusive staining of Nup88 or HBcAg. This inverse correlation between Nup88 and HBcAg expression patterns was confirmed by IHC double staining in which hepatocytes expressing HBcAg showed negative Nup88 expression and vice versa (data not shown).

Nup88 expression in hepatoma cell lines

To evaluate the impact of the presence of HBV on Nup88

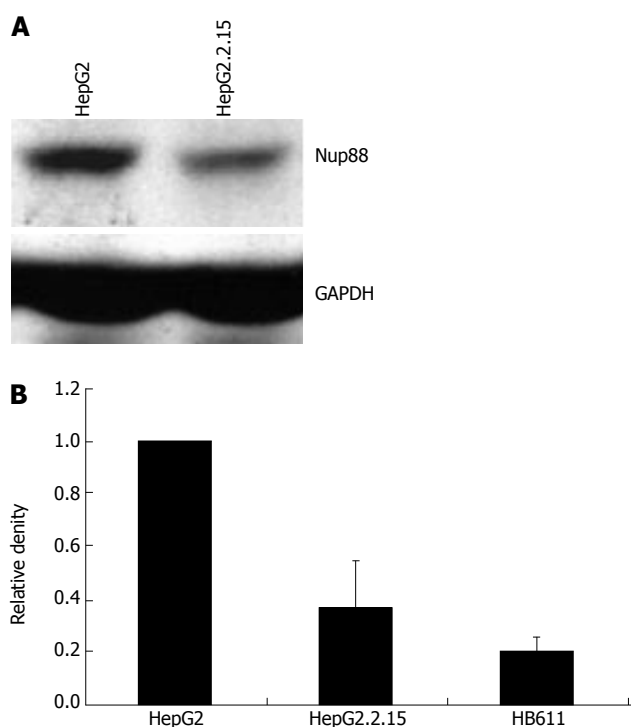


Figure 2 Western blot analysis showing reduced expression of Nup88 in HBV-positive HepG2.2.15 cells (A) and increased Nup88 expression in HBV-negative HepG2 and HBV-positive HepG2.2.15 ($P < 0.002$) and HB611 ($P < 0.001$) cells (B).

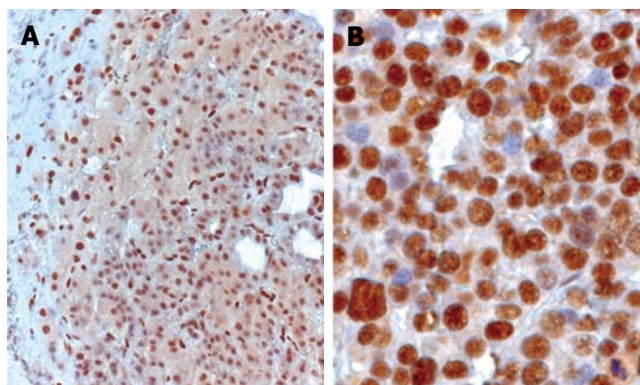


Figure 3 Nuclear Nup88-expression in a DN (A) and in dark brown nuclei of an undifferentiated HCC (B).

expression in cell culture the expression of Nup88 was assessed in hepatoma cell lines, i.e. the HBV-negative HepG2 and HBV-positive HepG2.2.15 and HB611 cell lines, by Western blot analysis. As expected, in HBV-negative HepG2 cells (Figure 2A) Nup88 expression could be clearly demonstrated. In contrast, Nup88 expression was significantly reduced in the HBV-positive HepG2.2.15 ($P < 0.002$) and HB611 ($P < 0.001$) hepatoma cell lines compared to HBV-negative HepG2 cells (Figure 2A and B).

Nup88 expression in dysplastic nodules and hepatocellular carcinomas

Nup88 was highly expressed in neoplastic hepatocytes of DN (Figure 3A) and HCC, but no significant differences were observed by IHC in Nup88 expression between

HBV- or HCV- associated HCCs and DN. Most if not all HCCs revealed a strong nuclear expression of Nup88. The strongest staining intensity was observed in undifferentiated HCC (Figure 3B). The high overexpression of Nup88 correlated with the degree of tumor cell dedifferentiation ($P < 0.0001$). In contrast, a weaker Nup88 expression was observed in DN. By comparing Nup88 expression intensity between DN and different grades of HCC, a significant increase was observed ($P < 0.0001$).

DISCUSSION

HCC is a major global health problem^[17]. The most common cause of HCC worldwide is chronic infection with HBV and HCV^[18]. Although the link of virus-related hepatocellular carcinogenesis has been well established, the underlying molecular pathogenesis is still poorly understood. In the present study we investigated the Nup88 expression in chronic viral hepatitis and virus-associated hepatocellular carcinogenesis. Nup88 is one of the known components of nucleoporins forming the nuclear pore complex (NPC) which is involved in nuclear transport. By using a polyclonal antiserum it has recently been shown that Nup88 is highly expressed in premalignant and malignant conditions^[7-11]. Here, we introduce a new monoclonal anti-Nup88 antibody which specifically detects Nup88 in Western-immunoblotting. In addition, this is the first monoclonal antibody to specifically detect Nup88 in paraffin-embedded and formalin-fixed tissues by IHC. In line with recently published data, our Nup88 antibody can also specifically detect neoplastic colorectal, mammary and lymphoma cells^[8,9]. Nup88 expression has recently been suggested as a marker for premalignant or malignant transformation. Therefore, the expression of Nup88 in non-neoplastic hepatocytes is remarkable. According to our results the expression of Nup88 in non-neoplastic liver tissue might reflect an elevated metabolic activity of cells that ensures the nuclear import and export of transcription factors^[2]. This hypothesis is supported by the finding of nuclear Nup88 expression in viral hepatitis as well as in HCC. In both cases hepatocytes exhibit an increased metabolic activity due to inflammation or malignant transformation. Until today Nup88 expression has only been described in the context of malignant transformation or tumor progression. Thus, our data might indicate additional functions of Nup88 expression in non-neoplastic liver tissue.

Reduced Nup88 expression in mild chronic HBV hepatitis compared to normal liver tissue and liver samples with mild uncharacteristic changes may contribute new insights to the understanding of HBV and NPC interaction. Recently, it has been shown that the HBV core protein binds to the NPC^[20,21]. However, the target domain of the anti-NPC antibody used in this study has not been exactly defined within the NPC, which consists of many different nucleoporins^[4,5]. We tempt to speculate that the low Nup88 detection in mild chronic HBV-associated hepatitis is due to an interaction of HBV with Nup88. The mechanism of this interference is not clear. However, it could occur directly by protein-protein interaction and therefore masking the Nup88 epitope or HBV may be

able to downregulate Nup88 at the transcriptional or protein level. Another hypothesis is that HBV, most likely the core antigen, might compete for NPC-components like CAN/Nup214. This is supported by the mutual exclusiveness of Nup88 and HBcAg expression in mild chronic HBV-associated hepatitis. In line with these data we have demonstrated in cell culture experiments that Nup88 expression is significantly reduced in HBV-positive HepG2.2.15 and HB611 cells compared to HBV-negative HepG2 cells. Moreover, recent work has identified the NPC-filament protein CAN/Nup214 as a docking site for incoming adenovirus type 2 (Ad2) capsid proteins^[22]. Therefore we hypothesize that NPC components like CAN/Nup214 and/or Nup88 might serve as docking sites for different viruses, e.g. HBV. Currently we are investigating not only this hypothesis in our laboratories by generating monoclonal Nup214 antibodies but also the possible transcriptional regulation of Nup88 by HBV. It is of interest that this phenomenon seems to be restricted to mild chronic HBV hepatitis and is not observed in later stages of hepatocellular carcinogenesis. Both increased nuclear Nup88 expression and elevated intensity correlate with the dedifferentiation of HCC and might be of interest in histopathological diagnostics. Although Nup88 expression in tumor cells is a marker for malignant transformation, it is highly overexpressed especially in undifferentiated HCC. Increased Nup88 expression has also been found in the colorectal adenoma/carcinoma sequence and overexpression of Nup88 mRNA has been shown to be associated with high aggressiveness of breast cancer^[3,9,10]. In contrast to Emlerling and coworkers^[10] we were not able to determine in our patient cohort whether the expression of Nup88 is of relevance with the survival of HCC patients, although undifferentiated HCCs tend to have a worse clinical outcome.

In summary, Nup88 is expressed in non-neoplastic and malignant hepatocytes, indicating that Nup88 plays a dichotomous role in non-neoplastic and neoplastic conditions of the liver. In addition, Nup88 is significantly overexpressed in poorly differentiated HCCs. The markedly reduced Nup88 expression in mild chronic HBV hepatitis and significantly less Nup88 expression in HBV-positive HepG2.2.15 and HB611 cells strongly point to an interference of HBV with the nuclear pore.

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Elevated plasma levels of N-terminal pro-brain natriuretic peptide in patients with chronic hepatitis C during interferon-based antiviral therapy

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Abstract

AIM: To investigate plasma levels of N-terminal pro-brain natriuretic peptide (NT-proBNP), an established marker of cardiac function, in patients with chronic hepatitis C during interferon-based antiviral therapy.

METHODS: Using a sandwich immunoassay, plasma levels of NT-proBNP were determined in 48 patients with chronic hepatitis C at baseline, wk 24 and 48 during antiviral therapy and at wk 72 during follow-up.

RESULTS: Plasma NT-proBNP concentrations were significantly increased ($P < 0.05$) at wk 24, 48 and 72 compared to the baseline values. NT-proBNP concentrations at baseline and wk 24 were closely correlated ($r = 0.8$; $P < 0.001$). At wk 24, 7 (14.6%) patients had NT-proBNP concentrations above 200 ng/L compared to 1 (2%) patient at baseline ($P = 0.059$). Six of these 7 patients had been treated with high-dose IFN- α induction therapy. In multiple regression analysis, NT-proBNP was not related to other clinical parameters, biochemical parameters of liver disease or virus load and response to therapy.

CONCLUSION: Elevated levels of NT-proBNP during and after interferon-based antiviral therapy of chronic hepatitis C may indicate the presence of cardiac dysfunction, which may contribute to the clinical symptoms observed in patients during therapy. Plasma levels of NT-proBNP may be used as a diagnostic tool and for guiding therapy in patients during interferon-based antiviral therapy.

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Key words: Hepatitis C; N-terminal pro-brain natriuretic peptide; Interferon; Cardiomyopathy; Treatment side effects

INTRODUCTION

The current standard of care for patients with chronic hepatitis C is the combination treatment with (pegylated) interferon plus ribavirin^[1,2]. Almost all patients experience side effects like fatigue, dyspnea and reduced physical activity. However, in many patients, these symptoms are not proportional to the decline of hemoglobin and resemble symptoms of heart failure.

Cardiotoxicity is assumed to be a rare complication of interferon therapy with few significant life-threatening cardiovascular effects reported^[3,4]. A small number of cases of suspected interferon-induced cardiomyopathy have been documented and in most of the patients, cardiac toxicity was reversible following the cessation of the drug therapy^[5].

Several studies have shown that plasma levels of brain natriuretic peptide (BNP) and N-terminal proBNP (NT-proBNP) are reliable diagnostic and prognostic markers for cardiac disease^[6,7] that correlate with symptoms of heart failure and the severity of systolic and diastolic dysfunction^[8]. In addition, BNP also predicts death, first major cardiovascular events, heart failure and stroke^[9].

In the present study, we assessed concentrations of circulating NT-proBNP in chronic hepatitis C patients before and during interferon-based antiviral therapy. Levels of this cardiac peptide were related to clinical, biochemical and virologic parameters in these patients.

MATERIALS AND METHODS

Subjects

The study population comprised of 48 patients (15 females and 33 males; mean age: 51 years, range: 33-76 years) with chronic hepatitis C virus infection. None of the patients had signs of heart failure, organic renal disease, thyroid disease, diabetes, cancer, or any other major diseases. All patients had normal cardiac physical examination and normal blood pressure. Thirty one patients were infected

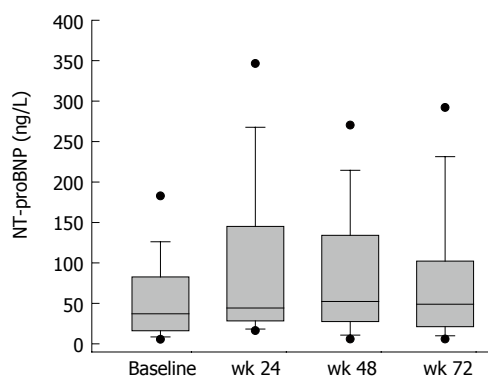


Figure 1 Plasma NT-proBNP concentrations before treatment, at wk 24 and 48 of therapy with interferon and ribavirin and at wk 72 of follow-up in patients with chronic hepatitis C virus infection (median with 5th/95th percentile; mean as a dotted line; Friedman repeated measures ANOVA, Student-Newman-Keuls method).

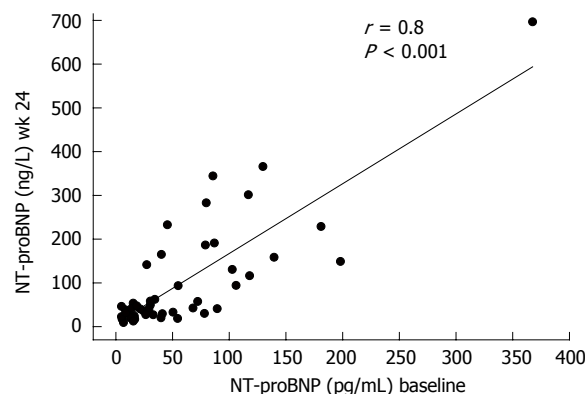


Figure 2 Correlation between plasma NT-proBNP concentrations before treatment and at wk 24 of therapy with interferon and ribavirin in patients with chronic hepatitis C virus infection (Spearman rank order correlation test).

with hepatitis C virus genotype 1, 6 with genotype 2, 10 with genotype 3 and 1 with genotype 4. All patients received interferon-based antiviral therapy for 48 wk. Sixteen patients received 3 Mio IU IFN-2b three times a week plus ribavirin 1000/1200 mg per day; 14 patients received 9 Mio IU IFN-2a per day for 2 wk, 6 Mio IU per day for 4 wk, 3 Mio IU per day for 6 wk and then 3x3 Mio IU per week plus ribavirin 1000/1200 mg per day; 11 patients received 10 Mio IU IFN-2b daily for 2 wk, then 5 Mio IU for 6 wk, 3 Mio IU for 16 wk and finally 3 Mio IU three times a week plus ribavirin 1000/1200 mg per day; 3 patients received 1.5 µg/kg PEG-IFN-2b for 4 wk, then 0.5 µg/kg plus 1000-1200 mg ribavirin; 2 patients received PEG 2a 180 µg plus 1000/1200 mg ribavirin; and 2 patients received PEG 2b 1.5 µg/kg plus ribavirin 800 mg daily.

Assays

All blood samples were drawn in the morning after an overnight fast. Blood samples for analysis of plasma NT-proBNP were collected, centrifuged and plasma was stored at -80°C until analysis. Plasma concentrations of NT-proBNP were measured by a sandwich immunoassay on an Elecsys 2010 (Roche Diagnostics, Mannheim, Germany). Blood samples were taken at baseline, at wk 24 and 48 during antiviral therapy, and at wk 72, i.e. 24 wk after the end of therapy.

Statistical analysis

Comparison of plasma NT-proBNP concentrations at baseline and during antiviral therapy and follow-up was performed with a Friedman repeated measures analysis of variance (ANOVA) on Ranks with an all pairwise multiple comparison procedure using the Student-Newman-Keuls method. Correlations were performed by the Spearman rank order correlation test. Fisher exact test was used to compare categorical variables. A multiple regression model was used to evaluate the relation between plasma NT-proBNP concentrations at baseline, during antiviral therapy and follow-up on the one hand and clinical, biochemical and virologic parameters on the other hand. Values were expressed as Mean ± SE. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Plasma NT-proBNP concentrations were significantly higher at wk 24 of therapy (mean 102 ± 18.4 ng/L; median 44.3 ng/L, range 9.2-696.4; *P* < 0.05), wk 48 of therapy (mean 89 ± 12.4 ng/L; median 52.4 ng/L, range 5.0-376.4; *P* < 0.05) and remained elevated in the follow-up period (mean 83 ± 14.1 ng/L; median 49.0 ng/L, range 5.0-433.3; *P* < 0.05) compared to baseline values before treatment (mean 59 ± 9.4 ng/L; median 37.1 ng/L, range 5.0-367.60; Figure 1). NT-proBNP concentrations at baseline and wk 24 were closely correlated (*r* = 0.8; *P* < 0.001; Figure 2). At wk 24, 7 (14.6%) patients had NT-proBNP concentrations above 200 ng/L compared with 1 (2%) patient at baseline (*P* = 0.059). Six of these 7 patients had received high-dose of interferon at the beginning of the treatment as an induction therapy, whereas 1 had received standard regimen with pegylated IFN once a week (*P* = 0.21).

In multiple regression analysis, plasma NT-proBNP concentrations before therapy were not related to other clinical or biochemical parameters of liver disease or virologic parameters and response to therapy (Table 1). However, elevated plasma NT-proBNP concentrations at wk 24 of therapy were predicted by plasma NT-proBNP concentrations before therapy (*P* < 0.01; Table 1).

DISCUSSION

The present study showed that circulating NT-proBNP concentrations increased significantly in patients with chronic hepatitis C virus infection during interferon-based antiviral therapy with a trend to affect patients more often that had received high-dose of interferon at the beginning of the therapy. This effect persisted during follow-up up to 24 wk after the end of therapy.

The findings of the present study may have important implications for patients with chronic hepatitis C virus infection during interferon-based antiviral therapy. Most patients complain about fatigue, dyspnea and reduced physical capacity during antiviral therapy which is frequently discontinued for these reasons. The pathogenesis of these symptoms is not well understood and sometimes attributed to the development of anemia. However, it seems possible that these patients experience

Table 1 Baseline characteristics of clinical, biochemical and virologic variables and their relations to baseline NT-proBNP concentrations in multiple regression analysis (mean \pm SE)

Variables	Value (mean \pm SE)	Regression coefficient	Standard error	P
Age (yr)	51 \pm 1.6	-0.03	0.8	0.96
Body weight (kg)	76.5 \pm 1.9	0.2	0.7	0.76
ALT (U/L)	59 \pm 5.9	0.3	0.34	0.35
AST (U/L)	31 \pm 2.9	-0.3	0.84	0.67
γ -GT (U/L)	41 \pm 6.1	-0.01	0.2	0.95
Bilirubin (mg/dL)	0.7 \pm 0.04	77	105.7	0.47
γ -globulins (mg/dL)	1.5 \pm 0.07	2.4	17.7	0.89
Prothrombin time (%)	95 \pm 1.5	0.7	1.0	0.51
Albumin (g/L)	4.8 \pm 0.04	-9.1	33.2	0.78
Cholinesterase (kU/L)	6.1 \pm 0.23	-1.4	6.1	0.81
Baseline HCV RNA (copies/mL)	4705302 \pm 984817	0.02 $\times 10^{-5}$	0.2 $\times 10^{-5}$	0.31
log decline of HCV RNA (baseline wk 12)	2.7 \pm 0.12	-5.9	16.0	0.71

cardiac impairment causing or at least contributing to these symptoms. Testing of NT-proBNP may serve as a screening marker for cardiac insufficiency in the differential diagnosis of fatigue and dyspnea and may alleviate the decision for further diagnostic testing of cardiac function as it has been described for other groups of patients^[10-13]. Besides diagnostic consequences, evaluation of NT-proBNP may have therapeutic consequences for patients with chronic hepatitis C virus infection during antiviral therapy with interferons as well. In patients with known congestive heart failure, elevated plasma BNP concentrations could be reduced by treatment with ACE inhibitors^[14], angiotensin II receptor antagonists^[15] as well as treatment with diuretics and vasodilators^[16]. As a consequence, plasma NT-proBNP concentrations may guide the intensity of pharmacotherapy as some interventional studies have suggested^[17,18].

In conclusion, this is probably the first study reporting elevated levels of NT-proBNP during and after interferon-based antiviral therapy in patients with chronic hepatitis C. This may indicate the presence of cardiac dysfunction and explain the clinical symptoms of patients during therapy and the discrepancy between these symptoms and the severity of anemia. Further prospective studies quantifying symptoms and correlating these with echocardiographic parameters are needed to confirm this association. In addition, interventional studies are required whether therapy of cardiac insufficiency can improve the side effect profile of interferon-based therapy in patients treated for chronic hepatitis C.

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RAPID COMMUNICATION

Sustained low diffusing capacity in hepatopulmonary syndrome after liver transplantation

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Abstract

AIM: To study the presence of sustained low diffusing capacity (DL_{CO}) after liver transplantation (LT) in patients with hepatopulmonary syndrome (HPS).

METHODS: Six patients with mild-to-severe HPS and 24 without HPS who underwent LT were prospectively followed before and after LT at mid-term (median, 15 mo). HPS patients were also assessed at long-term (median, 86 mo).

RESULTS: Before LT, HPS patients showed lower PaO_2 (71 ± 8 mmHg), higher $AaPO_2$ (43 ± 10 mmHg) and lower DL_{CO} ($54\% \pm 9\%$ predicted), due to a combination of moderate-to-severe ventilation-perfusion (V_A/Q) imbalance, mild shunt and diffusion limitation, than non-HPS patients (94 ± 4 mmHg and 19 ± 3 mmHg, and $85\% \pm 3\%$ predicted, respectively) ($P < 0.05$ each). Seven non-HPS patients had also reduced DL_{CO} ($70\% \pm 4\%$ predicted).

At mid- and long-term after LT, compared to pre-LT, HPS patients normalized PaO_2 (91 ± 3 mmHg and 87 ± 5 mmHg), $AaPO_2$ (14 ± 3 mmHg and 23 ± 5 mmHg) and all V_A/Q descriptors ($P < 0.05$ each) without changes in DL_{CO} ($53\% \pm 8\%$ and $56\% \pm 7\%$ predicted, respectively). Post-LT DL_{CO} in non-HPS patients with pre-LT low DL_{CO} was unchanged ($75\% \pm 6\%$ predicted).

CONCLUSION: While complete V_A/Q resolution in HPS indicates a reversible functional disturbance, sustained low DL_{CO} after LT also present in some non-HPS patients, points to persistence of sub-clinical liver-induced pulmonary vascular changes.

INTRODUCTION

Single-breath diffusing capacity (DL_{CO}) for carbon monoxide (transfer factor) impairment is the single most commonly abnormal lung function test in patients with end-stage hepatic disease^[1]. Although the mechanism of this isolated abnormality in advanced hepatic patients without chronic respiratory co-morbidities remains largely unknown, its high prevalence suggests sub-clinical liver-induced changes in the pulmonary vascular bed. Pathophysiologically, alveolar ventilation to pulmonary blood flow (V_A/Q) imbalance secondary to narrowing and early closure of airways to dependent lung zones as a consequence of interstitial edema and/or ascites in the context of fluid retention has been suggested as the most plausible mechanism^[1,2].

Alternatively, in patients with hepatopulmonary syndrome (HPS), an arterial oxygenation defect caused by intrapulmonary vascular dilatations (IPVD) in patients with liver disease^[3,4], V_A/Q mismatching along with intrapulmonary shunt (i.e., zero V_A/Q units) and diffusion limitation for oxygen essentially reflecting a diffusion-perfusion defect^[5] encompass the frequent finding of low DL_{CO} ^[4]. It is highly likely that in HPS DL_{CO} is reduced because the distance between the alveoli and the red cells in the central stream of the dilated pulmonary microvessels is too great for complete equilibration of CO with hemoglobin^[4]. Diffusion limitation for oxygen may be aggravated in part by a high cardiac output (Q_T) resulting in a shorter transit time of the red blood cell, hence contributing to the development of the diffusion-perfusion abnormality^[4].

We have shown that, in patients with HPS, several gas exchange markers, namely PaO_2 , alveolar-to-arterial PO_2

difference (AaPO₂), intrapulmonary shunt and increased low V_A/Q regions, correlate with DL_{CO}^[6]. Similarly, greater predicted (calculated according to the multiple inert gas elimination technique [MIGET]^[7]) than measured PaO₂, an indirect estimate of diffusion limitation for oxygen, correlates with DL_{CO} in hypoxemic HPS patients^[6]. Low transfer factor is also a reliable predictor for the diagnosis of HPS^[6].

Liver transplantation (LT) is the only therapeutic approach leading conclusively to complete resolution of advanced hepatic disease, including gas exchange abnormalities in HPS^[4,8-10]. Although sustained low DL_{CO} in hepatic patients with and without HPS has been reported after LT^[8,9,11,12], this finding remains controversial. Persistence of low DL_{CO} after LT would point to an underlying pulmonary vascular derangement.

We therefore decided to investigate comprehensively gas exchange status before and after LT in hepatic patients with and without HPS, to shed further light into the pathophysiology of low DL_{CO} in advanced hepatic disease states.

MATERIALS AND METHODS

Subjects

From our original cohort of 80 candidates for LT (14 patients with and 66 without HPS)^[6], a subset of 42 recipients of LT (53%) (9 with and 33 without HPS) between 1995 and 1997 was prospectively followed. An additional patient with HPS transplanted in 2000 was also included. From these 43 patients, 4 HPS (3 died before follow-up, 1 declined consent) and 9 non-HPS patients (6 died before follow-up, 3 refused to participate) were lost, the final population including 30 patients (6 with and 24 without HPS) (Table 1). Initial immunosuppressive therapy consisted of systemic corticosteroids, azathioprine and cyclosporine. Azathioprine was administered only during the first postoperative month. The dosage of corticosteroids was progressively tapered until discontinuation at the 18-24th mo. Cyclosporine was maintained through the period. The doses of these immunosuppressive drugs were also appropriately modified according to development of serious adverse effects or organ rejection. The study was approved by the Ethics Committee of our centre and all patients gave their written informed consent.

The diagnosis of HPS was based on the presence of an oxygenation defect [namely, increased AaPO₂ ≥ 15 mmHg while breathing air in upright position, irrespective of the presence of hypoxemia (PaO₂ < 80 mmHg)] and indicative evidence of IPVD [using contrast-enhanced echocardiography (CEE)] in a patient with underlying chronic hepatic disease, according to the European Respiratory Society (ERS) recommendations^[4].

Study design

Lung function tests were assessed in all patients before LT (median, 4 mo; range, 2 wk-18 mo; HPS patients, 2 wk-12 mo) and between 12 and 18 mo (median, 15 mo; mid-term ≤ 1.5 yr) following LT. Patients with HPS were also evaluated at long-term (≥ 3 yr after LT) (median, 86 mo;

Table 1 Physical and clinical characteristics of patients with and without hepatopulmonary syndrome (HPS)

Characteristics	HPS	Non-HPS
Patients (n)	6	24
Gender (F/M)	2/4	9/15
Age (yr)	43 ± 6	54 ± 2
Smoking Habits (n)		
Smokers	1	5
Ex-smokers	2	5
Etiology (n)		
Hepatitis C	3	12
Alcohol	1	4
Idiopathic	0	4
Other	2	4
Child-Pugh, n (%)		
A	1 (17)	3 (13)
B	4 (66)	13 (54)
C	1 (17)	8 (33)
Gastrointestinal bleeding, n (%)	4 (66)	10 (42)
Ascites, n (%)	3 (50)	18 (75)
Cutaneous spider naevi, n (%)	6 (100)	17 (71)
Concomitant respiratory Symptoms, n (%)		
Digital clubbing	4 (66)	2 (8) ^a
Dyspnea	6 (100)	3 (12) ^a
Cyanosis	2 (33)	0 (0) ^a

^aP < 0.05 vs non-HPS.

range, 45-117 mo) (one patient refused arterial puncture at this time point only). CEE measured as previously described^[6], was performed before LT in all patients and also after LT in HPS patients (at mid-term only). In the latter individuals, V_A/Q distributions were also estimated before and at mid-term after LT.

Measurements

Forced spirometry, static lung volumes, DL_{CO} after correction for anemia^[13], minute ventilation (V_E), arterial respiratory blood gases (PaO₂, PaCO₂, AaPO₂) and pH, and oxygen consumption (VO₂) were measured or calculated as previously described^[6]. In the 6 patients with HPS, V_A/Q distributions were calculated using MIGET as previously described^[6]. A pulmonary artery catheter was inserted (Swan-Ganz catheter, Baxter Healthcare, Irvine, CA, USA) in 4 patients and MIGET was computed using mixed venous respiratory and inert gases and cardiac output (Q_T) determined by thermodilution. In the remaining 2 HPS patients, Q_T was measured by dye dilution (DC-410; Waters Instruments, Rochester, MN, USA) such that mixed venous inert gases and PO₂ were estimated by mass balance in the customary manner^[7]. The agreement between these two MIGET approaches has been previously validated in our laboratory^[14]. The dispersion of the distributions of pulmonary blood flow (Log SDQ) and alveolar ventilation (Log SDV) on a logarithmic scale (upper normal limit, 0.60 and 0.65, respectively)^[15], key descriptors of the amount of low and high V_A/Q units respectively, and the difference among measured retentions and excretions of the inert gases corrected for the

Table 2 Lung function test and blood gas analysis before and after liver transplantation (LT) (mean \pm SE)

Parameter	HPS ¹			Non-HPS ¹	
	Pre-LT	Mid-term Post-LT	Long-term Post-LT	Pre-LT	Mid-term Post-LT
FEV ₁ ² (% pred)	89 \pm 7	92 \pm 8	87 \pm 9	90 \pm 4	89 \pm 4
FVC ³ (% pred)	97 \pm 6	100 \pm 7	93 \pm 11	89 \pm 3	88 \pm 3
FEV ₁ ² /FVC ³ 4 (%)	72 \pm 4	77 \pm 5	74 \pm 5	76 \pm 2	76 \pm 2
TLC ⁴ (% pred)	100 \pm 7	100 \pm 9	99 \pm 9	96 \pm 2	93 \pm 18
DLco ⁵ (mL/min/mmHg)	16.8 \pm 3.4 ^c	16.6 \pm 3.5	16.9 \pm 3.3	23.1 \pm 2.1	20.7 \pm 3.5
DLco ⁵ (% pred)	54 \pm 9 ^c	53 \pm 8	56 \pm 7	85 \pm 3	79 \pm 3
Kco ⁶ (%pred)	59 \pm 10 ^c	54 \pm 6	60 \pm 7	91 \pm 3	86 \pm 4
V _E ⁷ (l/min)	10.3 \pm 1.2	7.4 \pm 0.4 ^a	na ¹³	10.3 \pm 1.0	8.4 \pm 0.6
VO ₂ ⁸ (mL/min)	263.7 \pm 22.8	247.0 \pm 26.2 ^a	na ¹³	270.3 \pm 17.6	254.3 \pm 13.2
Hemoglobin (g/L)	111 \pm 22	121 \pm 20	134 \pm 21	104 \pm 18	114 \pm 16
PHa ⁹	7.45 \pm 0.02	7.39 \pm 0.01 ^a	7.42 \pm 0.01	7.47 \pm 0.01	7.41 \pm 0.01 ^a
PaO ₂ ¹⁰ (mmHg)	71 \pm 8 ^c	91 \pm 3 ^a	87 \pm 5 ^a	94 \pm 4	94 \pm 2
PaCO ₂ ¹¹ (mmHg)	28 \pm 2	36 \pm 1 ^a	34 \pm 0 ^a	31 \pm 1	35 \pm 1 ^a
AaPO ₂ ¹² (mmHg)	43 \pm 10 ^c	14 \pm 3 ^a	23 \pm 5 ^a	19 \pm 3	14 \pm 2

¹Hepatopulmonary syndrome; ²Forced expiratory volume at 1 s; ³Forced vital capacity; ⁴Total lung capacity; ⁵Single-breath carbon monoxide diffusing capacity; ⁶carbon monoxide diffusing capacity normalized per liter alveolar volume; ⁷Minute ventilation; ⁸Oxygen uptake; ⁹Arterial pH; ¹⁰Partial pressure of arterial oxygen; ¹¹Partial pressure of arterial carbon dioxide; ¹²Alveolar to arterial oxygen partial pressure gradient; ¹³Not available. ^a $P < 0.05$ vs pre-LT; ^c $P < 0.05$ vs non-HPS.

Table 3 Individual lung function test before and after liver transplantation (LT) in hepatopulmonary syndrome patients (% pred)

	Pre-LT						Mid-term post-LT						Long-Term Post-LT					
	1	2	3	4 ²	5 ¹	6 ²	1	2	3	4	5	6	1	2	3	4	5	6
Patients No.	106	110	77	90	71	80	106	120	74	83	96	81	102	123	76	71	66	87
FEV ₁	106	110	77	90	71	80	106	120	74	83	96	81	102	123	76	71	66	87
FVC	99	116	77	106	87	97	99	126	64	100	83	93	99	131	70	106	66	87
FEV ₁ /FVC	84	76	81	65	62	61	81	78	94	63	80	65	80	76	89	52	75	74
TLC	93	118	70	117	96	104	92	125	69	122	88	102	92	138	68	114	85	96
DLco	34	70	24	43	77	78	47	77	29	39	71	58	46	85	34	46	57	69

¹Smoker; ²Ex-smoker. For other abbreviations see Table 2.

elimination of acetone (DISP R-E*) (normal values < 3.0), an overall (mathematical) index of V_A/Q heterogeneity^[16], all dimensionless, were the most accurately used indices of V_A/Q imbalance. Intrapulmonary shunt and low V_A/Q regions were defined as the fraction of blood flow diverted to lung units with low V_A/Q ratios < 0.005 and between 0.005 and 0.1, respectively. Dead space was defined as the fraction of alveolar ventilation diverted to lung units with V_A/Q ratios > 100 .

Statistical analysis

Descriptive data are expressed as mean \pm SE. Paired and unpaired Student's *t* tests were used to compare data before and after LT and Pearson's correlations test was used when appropriate. $P \leq 0.05$ was considered statistically significant at all effects.

RESULTS

Before LT

Lung function tests before and after LT in patients with and without HPS are set out in Table 2. Respiratory symptoms and signs were more prevalent in HPS patients. Although mean spirometric and static lung volumes were within normal limits without differences between the two subgroups, individually one non-smoker with HPS (No.3)

had a mild restrictive pattern (due to pleural thickening) and 3 other HPS patients (No.4-6) (one smoker and two ex-smokers) had mild to moderate airflow limitation (Table 3). Compared to patients without HPS (Table 2), DLco and DLco/alveolar volume (Kco) were mildly to severely decreased ($< 80\%$ predicted) in each HPS patient ($P < 0.05$ each). In patients without HPS, DLco was mildly to moderately reduced ($70\% \pm 4\%$ predicted; range, $54\%-79\%$ predicted) in 7 (Figure 1), all but 2 (PaO₂, 58 and 74 mmHg each) normoxemic (PaO₂, 92 ± 8 mmHg; FEV₁, $93\% \pm 5\%$; FEV₁/FVC ratio, $78\% \pm 2\%$). In contrast, DLco was normal ($91\% \pm 3\%$ predicted; range, $80\%-131\%$ predicted; PaO₂, 95 ± 5 mmHg) in the 17 remainders without other differences between these two subgroups. Both V_E and VO₂ in all patients and Q_T, measured in HPS patients only, were increased. Chest high-resolution CT scans in HPS patients excluded the coexistence of emphysematous and/or diffuse interstitial changes.

Three HPS patients (No.1, No.3 and No.4) had severe disease (PaO₂ $< 60 - \geq 50$ mmHg), one (No.2) moderate (PaO₂ $< 80 - \geq 60$ mmHg) and the two remainders (No.5 and No.6) a mild stage (PaO₂ ≥ 80 mmHg; AaPO₂, 26 and 17 mmHg each)^[4] (Figure 1). Patients with HPS had lower PaO₂ and higher AaPO₂ than non-HPS patients ($P < 0.05$ each), while PaCO₂ was equally decreased in the two groups. Distributions of V_A/Q ratios were

Table 4 Ventilation-perfusion distributions in patients with hepatopulmonary syndrome before and after liver transplantation (LT) (mean \pm SE)

	Pre-LT	Post-LT
Shunt ¹ , % Q _T ²	7.8 \pm 3.3	0.6 \pm 0.1 ^a
Low V _A /Q mode ³ , % of Q _T	4.1 \pm 2.1	0.0 \pm 0.0 ^a
Mean Q ⁴	0.85 \pm 0.13	0.84 \pm 0.04
Log SDQ ⁵	0.95 \pm 0.17	0.49 \pm 0.08 ^a
Mean V ⁶	1.50 \pm 0.25	1.11 \pm 0.15
Log SDV ⁷	0.64 \pm 0.04	0.47 \pm 0.07 ^a
DISP R-E ⁸	11 \pm 2	4 \pm 1 ^a
Dead space, %	18.8 \pm 3.9	19.3 \pm 4.6
Pred-meas PaO ₂ ⁹ , mmHg	6 \pm 4	7 \pm 3

¹Percentage of blood flow to unventilated units (V_A/Q < 0.005) (normal, 0% of Q_T); ²Cardiac output; ³Perfusion to units with V_A/Q ratios between 0.005 and 0.1 (normal, 0% of Q_T); ⁴Mean V_A/Q of the perfusion distribution; ⁵Dispersion of blood flow distribution (normal < 0.60); ⁶Mean V_A/Q of the ventilation distribution; ⁷Dispersion of ventilation distribution (normal < 0.65); ⁸The difference among measured retentions and excretions of the inert gases corrected for the excretion of acetone (normal < 3.0); ⁹Predicted PaO₂ (by MIGET)-actual PaO₂ (measured). ^aP < 0.05 vs pre-LT.

abnormal in each HPS patient, as shown by mild to severe increases in Log SDQ and DISP R-E*, mild to moderate increases in intrapulmonary shunt and mildly increased low V_A/Q regions (Table 4). The mean distribution of pulmonary blood flow (mean Q) was mildly reduced (normal, ~1.0). By contrast, Log SDV was normal and the mean distribution of alveolar ventilation (mean V) was moderately increased. Dead space was severely reduced. In 3 hypoxemic HPS patients, predicted (according to MIGET) PaO₂ (68 \pm 2 mmHg) was greater than actual measured PaO₂ (55 \pm 2 mmHg). There was a negative correlation between the latter PO₂ difference and low DL_{CO} in all HPS patients ($r = -0.85$; $P < 0.05$), suggesting a close relationship between diffusion impairment for oxygen and DL_{CO}.

After LT

Liver function tests were within normal limits in all patients and diseases such as *de novo* autoimmune hepatitis, non-alcoholic steato-hepatitis, chronic rejection, and severe hepatitis C recurrence were excluded. At mid-term, all patients improved clinically while mean FEV₁ and lung volumes remained unchanged.

In patients with HPS, CEE-evidence for IPVD normalized, while both breathlessness and exercise tolerance substantially ameliorated or disappeared. Three HPS patients showed progressive resolution of finger clubbing. As opposed to the absence of any improvement in both DL_{CO} and K_{CO}, the three arterial blood gas markers substantially ameliorated reaching normal limits ($P < 0.05$ each) close to those of non-HPS patients before LT. This was essentially due to resolution of all V_A/Q descriptors, namely intrapulmonary shunt, regions of low V_A/Q units, Log SDQ and DISP R-E* ($P < 0.05$ each) (Table 4). Dead space and mean V remained unchanged. Predicted (98 \pm 2 mmHg) and actual (91 \pm 3 mmHg) PaO₂ in pre-LT hypoxemic HPS normalized and there was no correlation between the latter PO₂ difference and low DL_{CO}.

In patients without HPS, mean PaO₂, AaPO₂ and

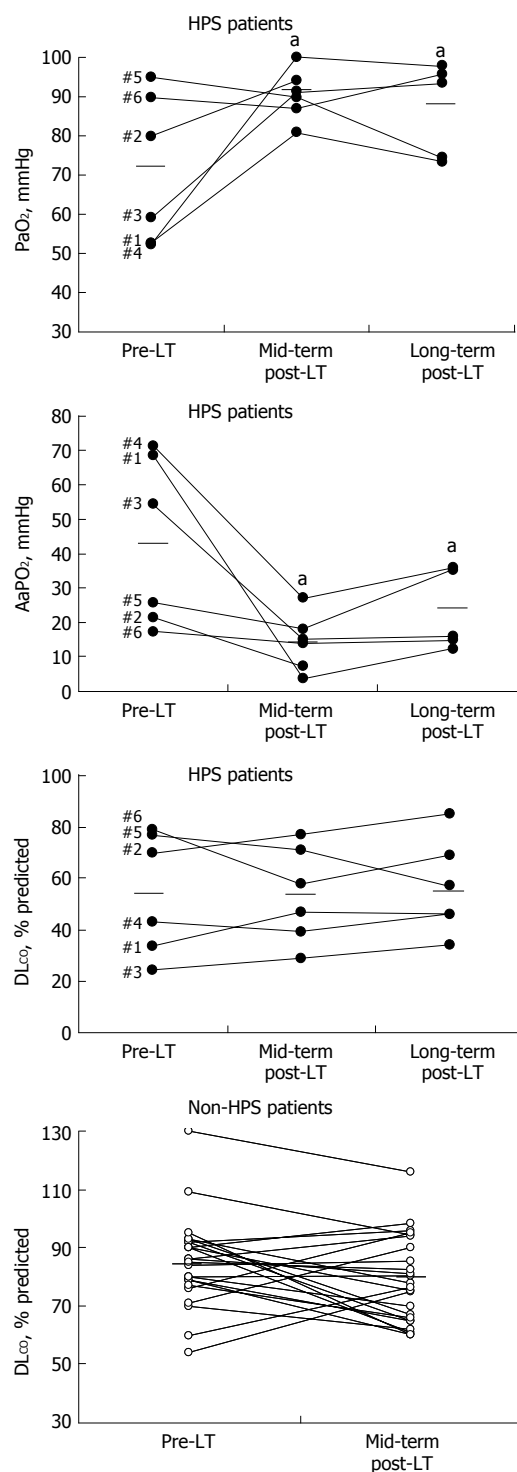


Figure 1 Individual values of arterial PO₂, AaPO₂ and DL_{CO} in patients with and without HPS before and after LT. Bold bars denote mean values. ^aP < 0.05 between pre- and post-LT measurements.

DL_{CO} remained unvaried (Table 2) including the DL_{CO} values of the 7 patients with pre-LT low DL_{CO} (75% \pm 6% predicted; range, 60%-95% predicted; PaO₂, 91 \pm 3 mmHg), in whom 2 had normalized DL_{CO}. Mean DL_{CO} in the remaining 17 non-HPS patients slightly decreased (to 81% \pm 4% predicted; range, 65%-116% predicted; PaO₂, 94 \pm 2 mmHg) ($P < 0.05$), whose physiological significance remained uncertain.

Mean PaCO₂ increased ($P < 0.05$ in HPS patients only) and arterial pH decreased ($P < 0.05$) in both HPS and non-

HPS patients, to reach normal limits without differences between the two populations. Increased PaCO₂ kept pace with decreased V_E, although only significantly in HPS patients ($P < 0.05$).

Pulmonary vascular resistance (PVR) increased (from 92 ± 69 dyn.s.cm⁻⁵ to 123 ± 78 dyn.s.cm⁻⁵), Q_T decreased (from 10.6 ± 4 to 6.6 ± 2 L/min) ($P < 0.05$ each) and pulmonary artery pressure was unchanged (from 18 ± 8 mmHg to 17 ± 6 mmHg) in the 4 patients with HPS in whom hemodynamics were carried out. Mean VO₂ decreased in HPS patients only ($P < 0.05$).

At long-term, 3 HPS patients had abnormal spirometry (Table 3): patient No.3 had a similar moderate restrictive pattern shown before LT, patient No.4 (ex-smoker) asymptomatic moderate airflow limitation possibly due to coexisting chronic obstructive pulmonary disease (COPD), and patient No.5 (smoker) mild-to-moderate undefined ventilatory pattern. Patient No.6 had normal spirometry. Unlike the persisting normalcy of arterial blood gases, mean DL_{CO} and K_{CO} in HPS remained unchanged at the same pre-LT levels (Figure 1).

DISCUSSION

The most salient finding of the present study is that all patients with HPS and a small subset of patients without HPS showed sustained reduced DL_{CO} as the most remarkable abnormal lung function test following successful LT. More importantly, in HPS patients these findings were observed in conjunction with normalization of all respiratory (PaO₂, PaCO₂ and AaPO₂) and inert gas exchange indices, namely regions with low V_A/Q units, increased dispersion of pulmonary blood flow (Log SDQ) and increased overall index of V_A/Q heterogeneity (DISP R-E*), intrapulmonary shunt and diffusion-perfusion defect (as shown by a greater predicted MIGET than actual measured PaO₂). Furthermore, most of the clinical, functional and hemodynamic abnormalities in HPS patients ablated or decreased and CEE-based IPVD disappeared. The absence of noticeable DL_{CO} changes after LT in non-HPS patients who received the same immunosuppressive therapy compared with patients with HPS, rules out at first glance any complementary iatrogenic interstitial lung effect. All in all, our findings point to persistence of sub-clinical structural changes at the pulmonary vascular bed.

Our results complement and extend a few prospective, although inconclusive and contentious, studies on the outcome of gas exchange disturbances after LT in patients with advanced hepatic disease^[8,9,11,12]. Eriksson *et al*^[8] observed in 6 patients with end-stage liver disease, but without proven-diagnosis of HPS, complete resolution of respiratory and inert gas exchange after LT, but they have not reported post-DL_{CO}. Krowka *et al*^[9] found that PaCO₂ and steady-state diffusing capacity are improved after LT without any reference to HPS. In contrast, Laberge *et al*^[11] reported that HPS is resolved in two children with HPS who normalized DL_{CO} and do particularly well after LT. It is postulated that, in juvenile HPS, the response of the pulmonary vasculature to LT appears to be more favorable than in adults^[17]. Finally, Battaglia *et al*^[12] have demonstrated

sustained low DL_{CO} after LT although improvement or resolution of hypoxemia and/or widened AaPO₂ is related to pulmonary capillary thickening^[18]. Notwithstanding, both the poor resolution of the gas exchange tools used and the lack of a solid definition of HPS in these studies have limited partially more evidence-based conclusions.

We are confronted therefore with the pathological basis of this intriguing sustained reduced DL_{CO} after LT in patients with and without HPS. We hypothesize that the persistence of low DL_{CO} after LT in patients with HPS may be consistent with structural pulmonary vascular changes already present before LT, such as increased thickness of the walls of small veins originally shown in a post-mortem case of liver cirrhosis complicated by cyanosis strongly suggestive of HPS^[19]. Ultrastructurally, this thickening is essentially due to a layer of collagen fibers interspersed with fine filamentous material whilst the basement membrane and many capillary walls are thickened with collagen, which provokes an approximately two-fold increase in the minimum blood-gas distance and contributes to the reduction in DL_{CO}^[19]. This structural vascular abnormality in HPS can be sub-clinical and still consistent with post-LT V_A/Q normalization and disappearance of intrapulmonary shunt and diffusion-perfusion defect. Persisting low DL_{CO} after LT in most of the subset of patients without HPS may be due either to a similar, albeit plausibly less severe, derangement than the one alluded to^[19] or alternatively to a different unknown abnormality of the pulmonary vascular bed as yet. Very recently, we have shown that pulmonary exchange defects in HPS patients remain unchanged after administration of N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) synthase, hence suggesting that HPS-induced gas exchange disturbances may be related to pulmonary vascular remodeling rather than to an ongoing vasodilator effect of enhanced NO production^[20].

Decline of DL_{CO} occurs after heart transplantation (HT) and persists for up to three years^[21], a finding that appears to contribute to exercise limitation in HT recipients^[22]. Post-HT DL_{CO} decline has been related to increased intra-capillary resistance to CO transfer secondary to a combination of anemia and reduced pulmonary capillary blood volume (Vc)^[23]. Post-HT normalization of pulmonary hemodynamics along with persisting structural pulmonary vascular changes due to pre-HT severe chronic heart failure may lie behind reduced Vc after HT^[23]. Notwithstanding severe pulmonary arterial hypertension in HT candidates is never present in patients with HPS. Accordingly, it may be unlikely that pulmonary hemodynamic changes after LT in our study might play a major role in post-LT persisting low DL_{CO}. Moreover, although three out of six HPS patients had an active (1) or past smoking (2) history, only one (patient No.4) had associated asymptomatic moderate airflow limitation that could explain, at least in part, persisting low DL_{CO} after LT. We have shown, however, that HPS-induced gas exchange disturbances and hemodynamic findings predominate in the face of coexisting chronic respiratory disorders, such as COPD^[24]. We did not assess the components of DL_{CO} because our primary aim was to ascertain in HPS patients whether or not pre-LT could reduce DL_{CO} persisted

within the context of gas exchange status following LT as compared to patients without HPS.

There are two strengths in our study, namely its prospective nature and lengthy design using clear-cut diagnostic criteria for patients with^[4] and without HPS, and the combined use of routine lung function tests along with one of the most robust tools to unravel the intrapulmonary determinants of arterial deoxygenation in HPS. We acknowledge, however, three limitations. The number of HPS patients studied is relatively small; the absence of pathological basis represents a weakness; and diffusion impairment cannot be estimated with MIGET when arterial hypoxemia is not present.

Persisting low DL_{CO} after LT in end-stage hepatic patients with and without coexisting HPS has not been previously reported in such a comprehensive manner. Whatever the ultimate mechanism for this unique and intriguing post-LT DL_{CO} impairment is, our findings point to the presence of pulmonary vascular involvement in advanced liver disease.

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RAPID COMMUNICATION

ECA39 is a novel distant metastasis-related biomarker in colorectal cancer

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CONCLUSION: Our results suggest that ECA39 is a dominant predictive factor for distant metastasis in patients with advanced CRC and that its suppression by PSK might represent a useful application of immunotherapy as part of a program of integrated medicine.

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Key words: ECA39; Distant metastasis; Colorectal cancer; Polysaccharide-K; Integrated medicine

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Abstract

AIM: To investigate the possible role of polysaccharide-K (PSK) -related markers in predicting distant metastasis and in the clinical outcome of colorectal cancer (CRC).

METHODS: Firstly, we used protein microarrays to analyze the *in vitro* expression profiles of potential PSK-related markers in the human colorectal adenocarcinoma cell line SW480, which carries a mutant *p53* gene. Then, we investigated the clinical implications of these markers in the prognosis of CRC patients.

RESULTS: ECA39, a direct target of c-Myc, was identified as a candidate protein affected by the anti-metastatic effects of PSK. Immunohistochemistry revealed that ECA39 was expressed at significantly higher levels in tumor tissues with distant metastases compared to those without ($P < 0.00001$). Positive ECA39 expression was shown to be highly reliable for the prediction of distant metastases (sensitivity: 86.7%, specificity: 90%, positive predictive value: 86.7%, negative predictive value: 90%). A significantly higher cumulative 5-yr disease free survival rate was observed in the ECA39-negative patient group (77.3%) compared with the ECA39-positive patient group (25.8%) ($P < 0.05$).

INTRODUCTION

Colorectal cancer (CRC) is one of the three most frequent malignancies in Western countries. CRC patient survival rates are delineated by local recurrence and lymphatic and hematogenous dissemination^[1,2] and, once metastatic disease is diagnosed, the 5-year survival rate is less than 5%. In the majority of cases, chemotherapy is the recommended treatment for patients with advanced metastatic disease. Recently, we have achieved good clinical results in the treatment of CRC using 'Pharmacokinetic Modulating Chemotherapy (PMC)', which was designed as a hybrid of lower metronomic and higher shorter plasma 5-FU concentration. The cumulative 5-year survival rate of Dukes' C CRC patients was 95% in the group treated with PMC, compared with 67% in the non-PMC group ($P = 0.003$)^[3,4]. Our PMC regimen also significantly decreased liver metastasis, resulting in a median liver metastasis-free time after hepatectomy of 34.2 mo in the PMC group compared with 18.4 mo in the non-PMC group ($P = 0.00002$)^[5]. PMC offers the advantages of reduced toxicity and lower costs through outpatient treatment. However, the prognosis of some patients remains poor despite vigorous anti-cancer therapy. Extrahepatic recurrence, mostly in the lung, cannot always be reduced by PMC alone, although PMC turned out well for the risk reduction of liver metastasis. Indeed, intensive chemotherapy has been shown to

cause a comparatively higher risk of extrahepatic distant metastases such as the lung, bone, brain and peritoneum, with an overall recurrence rate in CRC patients receiving PMC of 13.1%^[6]. While chemotherapy with hepatic arterial infusion or second-look liver resection can control the prognosis of liver metastasis to some extent, extrahepatic recurrence is refractory to any known treatment. The management of extrahepatic recurrence is a major problem as the prognosis of patients suffering extrahepatic recurrence is significantly worse (mean survival of 2 mo from diagnosis) compared with those with liver metastasis alone (mean survival of 26 mo from diagnosis) ($P < 0.05$)^[6].

The integration of immunopotentiating agents with the extant treatment regimens of surgery, chemotherapy, and radiation therapy has gained popularity as an adjuvant therapy for cancer during the last three decades. The use of complementary and alternative medicine (CAM) is a growing field in health care, particularly among cancer patients in the advanced stages of disease. However, recent reports have shown that while expenditure on CAM is high, 44.6%-66.7% of cancer patients receiving palliative care use CAM without sufficient information^[7,8].

Polysaccharide-K (PSK), or Krestin, is a protein-bound polysaccharide biological response modifier prepared from the mushroom *Coriolus versicolor*, that has been used in traditional Chinese medicine for centuries. PSK is widely used in adjuvant therapy after surgery or radiotherapy in Japan and other Asian countries, and the Japanese National Health Insurance scheme covers the use of PSK for gastric, colorectal, and lung cancers. Randomized, controlled clinical studies have revealed that the use of PSK in adjuvant therapy for gastric, colorectal, esophageal, and lung cancers significantly extends the 5-year survival rates of patients by 10% to 20%^[9-12]. Compared with CRC patients who did not receive PSK, PSK-treated patients showed a higher 5-year survival rate (73.0% in PSK group *vs* 58.8% in non-PSK group in stage II or III, and 60.0% *vs* 32.1% in stage III only) and lower rates of local recurrence (OR 0.74) and systemic recurrence (OR 0.52); lung metastases (OR 0.27), lymph node recurrence (OR 0.16), and peritoneal dissemination (OR 0.86)^[10]. Based on the findings of these studies, we have been administering PSK to patients with advanced CRC since 2001. Compatible with the findings of Ohwada *et al*^[10], we have observed that extrahepatic recurrences are significantly decreased when PSK therapy is used in combination with the standard PMC regimen (manuscript in preparation).

PSK produces very few adverse side effects, and its characteristics allow long-term oral administration. *In vitro* studies have confirmed that PSK induces the expression of several cytokine genes including *TNF- α* , *IL-1*, *IL-1R*, *IL-2*, *IL-4*, *IL-6*, *IL-7*, and *IL-8*^[12-15]. Anti-neoplastic effects of PSK have also been reported in animal models, and involve the radical trapping and modulation of cytokine production and effector cell functions^[16,17]. Recently, we have shown that PSK may have an additional anti-tumor effect on the cancer cells *per se* without disturbing cell-cycle progression^[18]. PSK might also alter the local characteristics of tissue-specific factors as well as their host-mediated

activities^[18].

We hypothesized that some PSK-related markers could be influential in predicting the occurrence of distant metastasis in CRC patients. In order to identify those molecular markers associated with the transition from primary CRC to distant metastases, we used a protein array containing 500 human antibodies in the human colorectal adenocarcinoma cell line SW480 to screen for alterations in protein expression that are potentially required for the direct action of PSK. SW480 carries a mutant *p53* gene; such mutations have been found in approximately half of all colorectal cancers and are associated with lymphatic dissemination and poor prognosis^[19,20]. We examined the expressions of candidate marker proteins in cancerous tissue obtained from CRC patients and accordingly assessed their usefulness as prognostic markers for distant metastasis.

MATERIALS AND METHODS

Cell culture and PSK treatment

The colorectal adenocarcinoma cell line, SW480, carrying a mutant *p53* gene was obtained from the Human Science Research Resource Bank (Tokyo, Japan). Cells were grown in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 100 mL/L fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 mmol/L glutamine, 100 000 U/L penicillin, 100 mg/L streptomycin, and 40 mg/L gentamycin at 37°C in a humidified atmosphere of 50 mL/L CO₂. For the cell growth study, 10⁶ cells were plated per 60-mm dish and treated with various concentrations of PSK (Kureha Chemical Co., Tokyo, Japan). Cells were counted using a hemocytometer on the days indicated. Then they were prepared for protein extraction and Ab arrays (Clontech, Palo Alto, CA, USA).

Identification of protein expression profiles by antibody microarray

Extraction of whole cellular protein, microarray hybridization, scanning, grid-assisted spot identification, and analysis were performed according to the manufacturer's instructions (Clontech). Briefly, 25 μ g whole cellular protein was extracted, labelled with the same volume of Cy3 and Cy5, hybridized with Antibody (Ab) Microarray, and the level of radioactivity was measured by scintillation counting. Sample and control-labelled probes were mixed together and hybridized to Ab Microarray slides containing 500 human antibodies in the Ab Microarray (No. 3080600). The names of these proteins are available at <http://www.clontech.com/clontech/products/families/abarray/nanoscale.shtml>. Hybridized slides were scanned and the scanner output images were analyzed using AtlasImageTM software, following localization by the overlaying of a grid on the fluorescent images. Fluorescent signal intensities were normalized by the Ab Microarray Analysis Workbook. Both final reported intensities were filtered, and those spots with intensities less than 0.75 or more than 1.32 were eliminated. The results were obtained from two independent experiments.

Patients

Sixty-three patients (31 women and 32 men) with a mean age of 60 years (range 37-83 years) and with surgically excised Dukes' C lower rectal carcinomas beneath the peritoneal reflexion were studied in the Hyogo College of Medicine between April 1986 and March 1995. Thirty-five of these patients (12 women and 23 men) with a mean age of 62.9 years (range 41-83 years) were enrolled in this study. The histological grades of carcinomas are as follows: 6 were well differentiated, 27 were moderately differentiated, and 2 were poorly differentiated or mucinous carcinomas. Follow-up information was obtained from office charts and hospital records. All patients were followed up for 60 mo after the initial operation. Local recurrence was defined as any tumor recurrence within the pelvis or anal canal. Distant recurrence was defined as any tumor recurrence outside the pelvis and included metastasis to the liver, lung, bone or the abdominal cavity. No recurrence was observed in 22 patients, distant recurrence was observed in 13 (3 cases of recurrence in the lymph nodes, 3 in the liver and 7 in the lungs), and local recurrence in 2 patients. The Ethics Committee of the Institution approved the study protocol.

Immunohistochemistry

CRC tissue specimens were processed using conventional procedures for paraffin embedding, cut into 4- μ m sections, and mounted onto poly-L-lysine-coated slides. Sections were dewaxed in xylene, rehydrated in a descending alcohol series, heated twice in a microwave oven for 5 min for antigen retrieval, blocked for endogenous peroxidase activity with 30 mL/L H₂O₂ in methanol, and then blocked for non-specific antibody binding with normal rabbit serum. They were incubated overnight at 4°C with a mouse monoclonal Ab against human ECA39 (BD Biosciences Pharmingen, San Jose, CA, USA) followed by treatment with a standard avidin-biotin-peroxidase complex. The slides were developed with 3, 3'-diaminobenzidine tetrahydrochloride solution containing 1 mL/L H₂O₂ and were lightly counterstained with hematoxylin. Normal mouse IgG was substituted for the primary antibody as a negative control. The sections were examined microscopically by three of the authors (Y.F., R.Y., and T.H.-T.) without knowledge of their clinicopathologic features. ECA39 expression was categorized according to staining intensity compared with interstitial infiltrates as follows: score 3 (strong), staining intensity more than interstitial infiltrates; score 2 (moderate), staining intensity equal to interstitial infiltrates; score 1 (mild), staining intensity less than interstitial infiltrates; and score 0 (negative), no staining. We then categorized ECA39 expression according to ECA39 expression scores: score 1 to 3, ECA39 positive; score 0, ECA39 negative.

Statistical analysis

Disease-free survival (DFS) and overall survival (OS) curves were generated by the Kaplan-Meier method, and the Cox-Mantel test was used to compare the curves. Death without recurrence was excluded from the analysis. Values of $P < 0.05$ were considered statistically significant. Statistical analyses were carried out using STATISTICA

Table 1 Differential protein expression in SW480 cell line following exposure to PSK, defined by a 1.32-fold or greater change

No.	Antidody/antigen name	Normalized average INR
102	HDJ-2	1.47
236	TNIK	1.46
376	TRAX	1.46
98	C-NAP1	1.35
225	Moesin	1.32
410	ECA39	0.65
403	Caspase-9/ICE-LAP6/Apaf-3	0.67
461	Synaptotagmin	0.71
406	ERK2 (MAPK2)	0.74
382	PMCA2	0.75

statistical software, version 06J (STATISTICA, Tulsa, OK, USA).

RESULTS

Suppression of cell growth by PSK

The effects of various concentrations of PSK (0 to 1000 mg/L) on the growth of SW480 cells was examined 96 h after treatment, and exposure to 10, 100, 500 and 1000 mg/L PSK was shown to suppress growth by 92.1%, 83.6%, 69.5% and 60.8%, respectively. Each figure represents the mean of more than three independent experiments.

Analysis of expression profiles

Protein expression in SW480 cells was analyzed following 24 h treatment with 500 mg/L PSK using an Ab Microarray (No. 3080600). Under basic selection conditions, a total of 10 proteins were selected from the 500 human proteins available on the array slide. These proteins were identified on the basis of their altered expression following exposure to PSK, with 1.32-fold or higher ratios, and included 5 up-regulated and 5 downregulated proteins (Table 1). Proteins showing upregulated expression, in the order of decreasing ratio, are HDJ-2, TNIK, TRAX, C-NAP1, and Moesin. Proteins showing downregulation, in the order of increasing ratio, are ECA39, Caspase-9/ICE-LAP6/Apaf-3, Synaptotagmin, ERK2 (MAPK2), and PMCA2.

Correlation of ECA39 expression with CRC patient prognosis

ECA39 was selected from these 10 candidate proteins as a distant metastasis-related marker in CRC after immunohistochemical analysis of CRC tissue specimens (Figure 1). ECA39 was expressed at significantly higher levels in tumor tissues with distant metastases (13 of 15 expressed positive ECA39) compared to those without metastases (2 of 20 expressed positive ECA39, $P < 0.00001$). Positive ECA39 expression was also shown to be highly reliable in predicting distant metastases (sensitivity: 86.7%, specificity: 90%, positive predictive value: 86.7%, and negative predictive value: 90%). Kaplan-Meier analysis revealed a significant

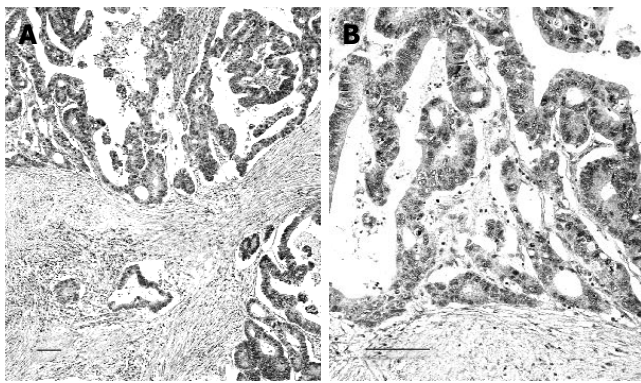


Figure 1 Immunohistochemical detection of ECA39 in rectal cancers (A: $\times 40$; B: $\times 100$. Bars indicate 100 μm).

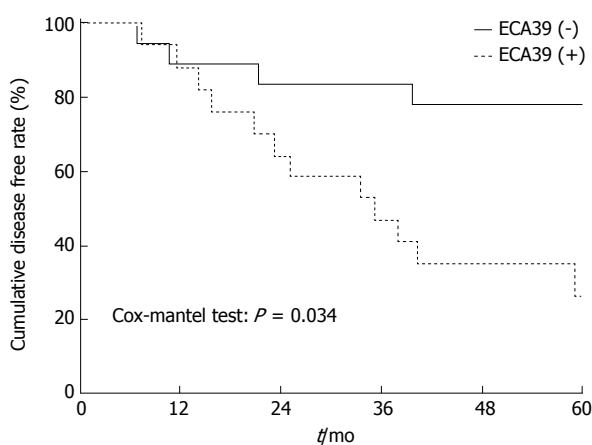


Figure 2 Five-year disease-free survival curves for eligible patients with pathologic stage III cancer in the ECA39-negative group and ECA39-positive group.

decrease in DFS of patients with positive ECA39 expression (25.8%) compared with patients with negative ECA39 expression (77.3%) ($P = 0.034$; Figure 2). The 5-year OS rate of ECA39-positive patients was 53.0%, compared with 83.3% for ECA39-negative patients, although this difference was not statistically significant ($P = 0.055$; Figure 3).

DISCUSSION

Normal cells undergo certain changes during their transformation into invasive malignant clones with metastatic potential. Molecular determinants occurring during the development of sporadic CRC include mutations in certain tumor suppressor genes (*APC*, *DCC*, *Smad-2*, *Smad-4*, *p53*) and oncogenes (*K-ras*) that have been summarized in the adenoma-carcinoma sequence initially proposed by Fearon and Vogelstein^[21]. However, because only 8% of CRC harbor concomitant mutations of *APC*, *K-ras*, and *p53*, it seems likely that additional pathogenic alterations are instrumental in the mediation of the progression and metastasis of CRC^[22]. Cellular transformation provokes tissue remodeling inside neoplastic lesions and in the periphery of the tumor. Disorders in the local characteristics of tissue-specific factors play an essential role in cancer

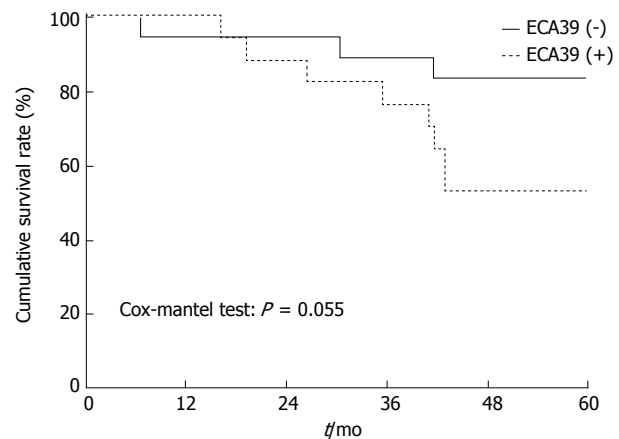


Figure 3 Five-year overall survival curves for eligible patients with pathologic stage III cancer in the ECA39-negative group and ECA39-positive group.

progression. In the present study, we have demonstrated that the ECA39 expression, which can be suppressed by PSK, has the potential to predict distant metastasis.

The ECA39 protein was originally identified by the overexpression of its mRNA in an undifferentiated mouse teratocarcinoma cell line^[23]. The *ECA39* gene harbors a functional *c-Myc* binding sequence located 3' of its transcription initiation site, and has been shown to be a direct target for c-Myc activity in both mice and humans^[24,25]. The functional implications of individual *c-Myc* target genes including ornithine decarboxylase^[26,27], *p53*^[28], and *cdc25A*^[29], are now complemented by large surveys of the c-Myc network as a therapeutic target in cancer^[30]. The *c-myc* oncogene is essential for cell proliferation but, paradoxically, also promotes cell death. The biological rationale for this dual signal is that c-Myc intrinsically regulates malignant transformation. ECA39 shares significant homology with the prokaryotic protein branched-chain amino acid aminotransferase (BCAT), and is highly expressed during the log phase and is down-regulated during the stationary phase of growth^[31]. Thus, ECA39 might be involved in the regulation of the cell cycle. Disruption of the *ECA39* gene results in an increased growth rate in comparison to wild type^[25]. As shown in the present study, ECA39 is a highly reliable marker in the prediction of distant metastasis and, in combination with other biomarkers, might produce an even higher predictability of poor prognosis. Despite significant progress in the identification of markers predicting CRC patient prognosis, there remains a need for clinical predictors of distant metastasis in order to strengthen patient surveillance. This would allow the tailoring of treatment to individual patients and the application of evidence-oriented integrated medicine, thus maximizing the probability of optimal response to the therapy.

Previous reports have demonstrated that c-Myc acts as a biomarker in the prediction of patient response to treatment with 5-FU and camptothecin^[32,33]. Evidence of ECA39-based administration of PSK could also favor some CRC patients by sensitizing their response to chemotherapy, protecting normal cells during treatment as well as having an anti-metastatic effect. Such possibilities need to be confirmed in larger clinical studies, which are war-

ranted both in Japan and world-wide. We believe that the acquisition of knowledge of integrated medicine by physicians, especially oncologists, is essential and should not be underestimated. Moreover, oncologists should discuss the role of integrated medicine with their patients and encourage patients to participate in well-organized research on integrated approaches to therapy.

Here, we identified ECA39 as a biomarker that predicts distant metastasis in CRC patients. ECA39 is thought to play a role in metastasis, and could represent a potential diagnostic, prognostic, or even therapeutic target. Furthermore, the metastasis-tumor associated ECA39 profile could be of use in the selection process of tumors that are likely to develop metastases, thus optimizing the application of immunotherapy by PSK and improving clinical outcome.

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RAPID COMMUNICATION

Organisms causing spontaneous bacterial peritonitis in children with liver disease and ascites in Southern Iran

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Abstract

AIM: To determine the causative agents of spontaneous bacterial peritonitis (SBP) in children with liver disease and ascites in our center.

METHODS: During a 2.5 year period, from September 2003 to March 2006, 12 patients with 13 episodes of SBP were studied. In all cases at the time of admission serum albumin and glucose, urinalysis and urine culture was performed. Analysis [white blood cell (WBC) count with differential, albumin, glucose], gram stain, culture by BACTEC method and antibiogram was done on ascitic fluids. Abdominal paracentesis was repeated after 48 h of antibiotic therapy for bacteriologic assay. The patients were followed for at least three months in a gastroenterology clinic.

RESULTS: There were 7 girls (58%) and 5 boys (42%) with a median age of 5.2 years (range, 6 mo to 16 years). All cases had positive ascitic fluid culture. Gram stain was positive in 5 (38.5%) of them. The isolated organisms were *S. pneumoniae* in 5 (38.5%), *E. coli* in 2 (15.3%), *S. viridans* in 2 (15.3%), and *K. pneumoniae*, *H. influenza*, *Enterococci*, and nontypable *Streptococcus* each in one (7.7%). All of them except *Enterococci* were sensitive to ciprofloxacin and ceftriaxone. All ascitic fluid cultures were negative after 48 h of antibiotic therapy.

CONCLUSION: *S. pneumoniae* is the most common cause of SBP in the pediatric age group and we recommend a third generation cephalosporine (e.g., Ceftriaxone or Cefotaxime) for empirical therapy in children with SBP.

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Key words: Spontaneous bacterial peritonitis; Children;

INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is defined as an ascitic fluid infection without a demonstrable intra-abdominal cause^[1]. It is a well known complication of cirrhosis in adults, occurring in 8% to 13% of patients^[2-5]. The diagnosis is established by a positive ascitic fluid bacterial culture and an elevated ascitic fluid absolute polymorphonuclear leukocyte (PMN) count ($\geq 2.5 \times 10^5/L$). In adults, the organisms of SBP are usually gram-negative bacteria^[2-4,6], but they may differ in children^[5,7]. The aim of the present study was to determine the causative agents of SBP in children with liver disease and ascites in our center.

MATERIALS AND METHODS

During a period of 2.5 years, from September 2003 to March 2006, 63 children with liver disease and ascites were prospectively studied in the Department of Pediatric Gastroenterology in Nemazee Hospital affiliated with Shiraz University of Medical Sciences, the major referral center in Southern Iran. Written consent was obtained from all parents after informing them about this study which was approved by the Ethics Committee of the University.

Of the children involved in this study 12 had met the criteria for SBP (polymorphonuclear leukocyte count greater than $2.5 \times 10^5/L$ and positive ascitic fluid culture). In total 13 episodes of SBP were documented, of which two occurred with one patient, 4 mo apart. The patients and their parents answered a structured questionnaire, which included name, age, sex, clinical history, underlying liver diseases, history of antibiotic and diuretic use, history of previous variceal bleeding and episodes of SBP. All children had undergone a thorough physical examination.

Serum albumin and glucose, urinalysis and urine culture were done in all patients. Paracentesis was performed

Table 1 Results of ascitic fluid cultures in 12 patients with SBP

Organism	n	%
<i>S. pneumoniae</i>	5	38.5
<i>E. coli</i>	2	15.3
<i>S. viridans</i>	2	15.3
<i>K. pneumoniae</i>	1	7.7
<i>H. influenza</i>	1	7.7
<i>Enterococci</i>	1	7.7
Nontypable streptococcus	1	7.7

on all patients under sterile conditions and analysis of fluid (Albumin, glucose, WBC count and differential) and gram stain was done. Ten milliliter of ascitic fluid was inoculated at the bedside in a blood culture bottle (BACTEC, PEDS PLUS/F medium, Becton, Dickinson Co. USA) using the BACTEC 9240 system (Becton, Dickinson Co. USA). Antibigram was performed with the disk diffusion method (Kirby-Bauer method) for ciprofloxacin (5), cefotaxime (30), ceftriaxone (30), gentamicin (10) and cotrimoxazole (1.25-23.15) (MAST Co. UK). Intravenous ceftriaxone (100 mg per kilogram body weight) was started empirically for all patients after paracentesis, as recommended in adult series^[8,9]. After 48 h of antibiotic therapy ascites were resolved in 4 patients. We could not obtain ascitic fluid despite sonographic guidance. One patient expired. Abdominal paracentesis was repeated in 8 cases for analysis and culture. The patients were followed for at least three months in our gastroenterology clinic.

RESULTS

Of the patients involved in this study, 27 had normal ascitic fluid analysis and negative cultures. In 22 patients fluid analysis showed greater than 2.5×10^5 per liter, but fluid cultures were negative. These patients were treated as culture negative neutrocytic ascites and were excluded from this study. Two patients had normal ascitic fluid analysis, but ascitic fluid cultures were positive for staphylococcus coagulase positive and negative, which were considered as non-neutrocytic Bacterascites. These were excluded from this study.

Of the SBP patients, there were 7 girls (58.3%) and 5 boys (41.6%) with a mean age of 5.2 years (range, 6 mo to 16 years). The most common clinical manifestations were as follows: fever in 12 (92.3%), abdominal pain in 12 (92.3%), abdominal tenderness in 12 (92.3%), change in level of consciousness in 5 (38.5%) and decreased bowel sounds in 3 (23%). Urinalyses were normal and urine cultures were negative in all patients. Serum-ascitic fluid albumin gradient was greater than 11 g/L in all (100%) patients. Total protein concentration of ascitic fluid was less than 10 g/L in 11 (85%) patients. Ascitic fluid glucose was greater than 0.5 g/L in all but one. Gram stains of ascitic fluid were positive in five patients (38.5%), including four gram positive cocci and one gram negative bacilli. Results of BACTEC cultures are shown in Table 1. The most common organism of SBP in this study was *Streptococcus*

Table 2 Ascitic fluid analysis on admission and after 48 h of antibiotic therapy in SBP patients

Patient No.	WBC ¹ count on admission	WBC ¹ count after 48 h	Rate of WBC ¹ decline (%)	PMN ² count On admission	PMN ² count after 48 h	Rate of PMN ² decline (%)
1	6600	3000	0.45	5940	1200	0.44
2	1400	500	0.36	910	200	0.61
3	600	550	0.92	480	165	0.37
4	950	140	0.15	684	56	0.55
7	4000	3200	0.80	3000	704	0.29
10	3680	85	0.02	3496	70	0.86
12	840	100	0.12	689	53	0.65
13	1000	78	0.08	800	37	0.60

¹ White blood cell; ² Polymorphonuclear leukocyte.

pneumoniae (38.5%). All organisms except *Enterococci* and one case of *E. coli* were sensitive to cefotaxime, and all of them except *Enterococci* were sensitive to ceftriaxone and ciprofloxacin. All patients became asymptomatic after 72 h of antibiotic therapy, except one infant with diagnosis of neonatal hepatitis and cirrhosis, who expired after 48 h due to encephalopathy. Abdominal paracentesis repeated in 8 cases after 48 h of antibiotic therapy. The results are shown in Table 2. Total WBC declined to 30% of primary counts, and PMN count halved after 48 h. All ascitic fluid cultures were negative after 48 h of antibiotic therapy.

DISCUSSION

Since the first descriptions of SBP by Kerr *et al* in 1963^[10] and Conn and Fessel in 1964^[11], the clinical presentation, treatment and prognosis of this disease have been well established. However, other aspects are still subject to investigation such as its pathogenesis^[12], diagnosis^[13] and prevention^[14].

Although most episodes of SBP occur in patients with advanced cirrhosis with ascites, occasionally it has been observed in non-cirrhotic patients such as fulminant hepatic failure^[15], nephrotic syndrome^[16] and congestive heart failure^[17]. SBP is a serious infection in patients with ascites, and it is the largest abscess in humans^[18]. To date, most studies of SBP have been done in adults with cirrhosis. The lack of reports focusing on pediatric patients is remarkable^[19]. The prevalence of SBP in our patients was 20.6%, which is relatively similar to Vieira *et al* study (19.5%)^[19]. The mean age of our cases was 5.2 years (6 mo to 16 years), which is relatively similar to the Larcher *et al* series (5.5 years)^[7]. Fever and abdominal pain were the most common clinical presentations in our cases, which is also similar to previous reports^[2-4,7]. In all cases in our study, urinalysis was normal and urine cultures were negative, so we conclude that there is no association between SBP and urinary tract infections. This result differs from the Hoefs *et al* study^[20] which concluded that the urinary tract can be a source of infection in SBP patients. The high incidence of pneumococcal infection (38.5%) in our study, which was lower than that reported by Larcher *et al* (75%)^[7],

distinguishes our series from most adult series^[2-4,6,21]. In our series there were 9 cases (69%) of gram positive and 4 cases (31%) of gram negative organisms, which is different from the adult series^[2-4,6]. In this study all organisms except one *Enterococci* were sensitive to ceftriaxone and ciprofloxacin.

In summary, Gram positive organisms are a more common cause of SBP than gram negative organisms in cirrhotic children in our area and a third generation of cephalosporin such as ceftriaxone or cefotaxime can be a suitable antibiotic for empirical therapy of children with SBP. We suggest that pneumococcal vaccination may play a role in the prevention of SBP in children, but its efficacy can only be established by further studies.

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Comparison of different diagnostic methods in infants with Cholestasis

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Abstract

AIM: To evaluate different methods in differentiating idiopathic neonatal hepatitis from biliary atresia.

METHODS: Sixty-five infants with cholestatic jaundice and final diagnosis of idiopathic neonatal hepatitis and biliary atresia were studied prospectively from September 2003 to March 2006. A thorough history and physical examination were undertaken and the liver enzymes were examined. All cases underwent abdominal ultrasonography, hepatobiliary scintigraphy, and percutaneous liver biopsy. The accuracy, sensitivity, specificity and predictive values of these various methods were compared.

RESULTS: There were 34 girls and 31 boys, among them 46 subjects had idiopathic neonatal hepatitis (age, 61 ± 17 d) and 19 had biliary atresia (age, 64 ± 18 d). The mean age at onset of jaundice was significantly lower in cases of biliary atresia when compared to idiopathic neonatal hepatitis cases (9 ± 13 d vs 20 ± 21 d; $P = 0.032$). The diagnostic accuracy of different methods was as follows: liver biopsy, 96.9%; clinical evaluation, 70.8%; ultrasonography, 69.2%; hepatobiliary scintigraphy, 58.5%; and liver enzymes, 50.8%.

CONCLUSION: Our results indicate that clinical evaluation by an experienced pediatric hepatologist and a biopsy of the liver are considered as the most reliable methods to differentiate idiopathic neonatal hepatitis and biliary atresia.

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Key words: Idiopathic neonatal hepatitis; Biliary atresia; Clinical evaluation; Liver biopsy

INTRODUCTION

Cholestatic jaundice in early infancy is an important clinical condition that results from diminished bile flow and/or excretion, and can be caused by a number of disorders. Idiopathic neonatal hepatitis (INH) and biliary atresia (BA) are two main causes^[1,2].

It is important to distinguish INH from BA in an infant presented with jaundice, as the former purely needs a medical management and the later requires surgical intervention as soon as possible. Therefore, rapid and accurate differentiation is crucial for early surgery in patients with BA^[3]. In this study, we evaluated and compared the different diagnostic methods for this differentiation.

MATERIALS AND METHODS

In a prospective study from September 2003 to March 2006, the differential diagnosis and etiologic work-up of cholestasis in infancy were carried out. A written consent was provided from all parents after informing them about this study which was also approved by the Ethic Committee of the University. Sixty-five consecutive cholestatic infants (34 girls, 31 boys) with a final diagnosis of INH or BA were entered in our study. These patients were all referred to the Department of Pediatric Gastroenterology in Nemazee Hospital affiliated with Shiraz University of Medical Sciences, which is the major referral center in Southern Iran.

A thorough history and physical examinations were provided, including age at onset of jaundice, birth weight, stool color, and any signs of systemic diseases. Ophthalmologic, cardiac and rectal examination, presence of any organomegaly, and assessment of growth state were performed.

For all patients, a complete blood count, urinalysis, urine reducing substances, thyroid function tests, bacterial culture of both urine and blood, serum alpha-1-antitrypsin and screening for cystic fibrosis (sweat chloride test) were performed. Acid-base status was determined as

Table 1 Comparison of various diagnostic methods for INH and BA

Method	Patients (n)	Results	Final diagnosis				P ¹
			BA (n = 19)		INH (n = 46)		
			n	%	n	%	
Clinical evaluation	65	BA	16	84.2	16	34.8	< 0.001
		INH	3	15.8	30	65.2	
Liver enzymes	65	BA	13	68.4	26	56.5	0.373
		INH	6	31.6	20	43.5	
Ultrasonography	65	BA	10	52.6	11	23.9	< 0.05
		INH	9	47.4	35	76.1	
Hepatobiliary scintigraphy	65	BA	16	84.2	24	52.2	< 0.05
		INH	3	15.8	22	47.8	
Liver biopsy	65	BA	19	100	2	4.3	< 0.001
		INH	0	0	44	95.7	

¹BA vs INH, κ^2 analysis.

Table 2 Accuracy of various diagnostic methods for BA and INH

Diagnostic method	n ¹	BA (%)	n	INH (%)	n	INH vs BA (%)
Liver biopsy	19/19	100	44/46	95.2	63/65	96.9
Clinical evaluation	16/19	84.2	30/46	65.2	46/65	70.8
Ultrasonography	10/19	52.6	35/46	76.1	45/65	69.2
Hepatobiliary scintigraphy	16/19	84.2	22/46	47.8	38/65	58.5
Liver enzymes	13/19	68.4	20/46	43.5	33/65	50.8

¹Accurately diagnosed cases/performed cases.

an initial step to evaluate the metabolic disorders.

For all patients, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were checked and all underwent ultrasonography of the abdomen, hepatobiliary scintigraphy and percutaneous liver biopsy.

Cases with etiologies other than INH and BA were excluded from the study. Cases suspicious for BA underwent laparotomy and intraoperative cholangiography.

Later onset of jaundice, lower birth weight, poor growth, deep yellow or greenish stools, presence of signs of systemic diseases and consanguinity were considered as clinical criteria for diagnosis of INH. ALT and AST 10 times more than normal mean and ALP 5 times less than normal mean are considered as criteria for INH. ALT and AST 5 times less than normal mean and ALP 5 times more than normal mean are considered as criteria for BA. Ultrasonographic evidences of dilatation or absence of the main bile duct near the hilum, absence of the gall bladder and presence of triangular cord sign were considered as criteria for BA.

Finally, the accuracy of the five diagnostic methods (clinical evaluation, liver enzymes, abdominal ultrasonography, hepatobiliary scintigraphy, and liver biopsy) was evaluated for differential diagnosis of INH and BA. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of each method were also calculated.

RESULTS

There were 65 cholestatic infants, including 34 (52.3%) girls and 31 (47.7%) boys with a mean age of 62 ± 17 d (30-91 d). There were 46 (70.8%) cases (22 girls, 24 boys) of INH, and 19 (29.2%) cases (12 girls, 7 boys) of BA. The mean age of INH patients was 61 ± 17 d (33-90 d), and BA patients was 64 ± 18 d (30-91 d) and the difference was not statistically significant.

Mean birth weight of patients with INH was 2893 ± 629 g (1150-3900 g) and with BA was 2951 ± 556 g (1900-4150 g), and the difference was not significant.

Age at onset of jaundice in INH was 20 ± 21 d (1-65 d) and in BA was 9 ± 13 d (1-45 d), and the difference was significant ($P = 0.032$).

Forty-four (67.7%) cases had clay-colored stools, among them, 26 cases had INH and 18 cases had BA; and 21 (32.3%) cases had normal-colored stools, among them, 20 cases had INH and one had BA ($P = 0.003$).

The difference in mean ALT, AST and ALP values between INH and BA patients was not significant.

Table 1 shows comparison of the various methods in diagnosing of 65 infants with cholestasis. Table 2 shows the diagnostic accuracy of each method in the order of accuracy. Liver biopsy had 100% diagnostic accuracy for BA and 95.2% for INH, and clinical evaluation had diagnostic accuracy of 84.2% and 65.2% for BA and INH, respectively. Table 3 shows the sensitivity and specificity and Table 4 demonstrates the positive and negative predictive values of each method in differentiating BA and INH. Liver biopsy had the highest sensitivity and specificity for differentiating BA and INH.

DISCUSSION

The North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition (NASPGHAN) guideline for the evaluation of cholestatic jaundice in infants recommends that any infant noted to be jaundiced at the two-week well child visit should be evaluated for cholestasis^[1]. Evaluation of breast-fed infants may be delayed until three weeks of age if they have a normal

Table 3 Sensitivity and specificity of various diagnostic methods for BA and INH

Diagnostic method	Sensitivity for BA		Specificity for BA	
	%	<i>n</i>	%	<i>n</i>
Clinical evaluation	84.2	16/19	65.2	30/46
Liver enzymes	68.4	13/19	43.5	20/46
Ultrasonography	52.6	10/19	76.1	35/46
Hepatobiliary scintigraphy	84.2	16/19	47.8	22/46
Liver biopsy	100	19/19	95.7	44/46

Sensitivity for BA was equivalent to specificity for INH, and specificity for BA was identical to sensitivity for INH.

physical examination, no history of dark urine or light stools, and can be reliably monitored^[1,4,5]. Neonatal hepatitis and BA, which typically occur in term infants, account for 70%-80% of cases^[6].

Evaluation should be undertaken in a staged approach^[7]. The initial step is rapid diagnosis and early initiation of therapy of treatable disorders. Conditions, such as sepsis, hypothyroidism, panhypopituitarism, and inborn errors of metabolism (e.g., galactosemia), must be recognized and treated promptly to avoid significant progression of the illness. Extrahepatic biliary atresia must be differentiated from neonatal hepatitis because early surgical intervention (i.e., before two months of age) results in a better outcome.

In our study, clinical evaluation had good accuracy for diagnosing BA (84.2%) and moderate accuracy for INH (65.2%). Prevalences of acholic stools in our study were 94.7% for BA and 56.5% for INH that are comparable to the study by Mowat *et al.*^[2] (i.e., 83% and 52%, respectively). The mean values of ALT, AST and ALP in BA were 161 ± 107 , 261 ± 141 , and 2150 ± 830 , respectively, and in INH were 212 ± 198 , 324 ± 258 , and 1791 ± 852 , respectively, and the difference was not statistically significant. Therefore, considering the level of liver enzymes would not be an accurate method to differentiate BA and INH (diagnostic accuracy of 50.8%).

Abdominal ultrasonography is more helpful in the diagnosis of choledochal cysts but can also suggest the diagnosis of BA. Findings suggestive for the latter are non-visualized gall bladder and the presence of the triangular cord sign^[8-10]. The sensitivity and specificity of a small or absent gall bladder in detecting obstruction range from 73% to 100% and 67% to 100%, respectively, when correlated with pathologic, surgical, and subsequent clinical examinations^[1].

In our study, abdominal ultrasonography had sensitivity and specificity of 52.6% and 76.1% for BA, respectively. Accuracy of ultrasonography for differentiation between BA and INH in our series was 69.2%, that was lower than that reported by Lin *et al.*^[11] (sensitivity, specificity and accuracy were 86.7%, 77.1%, and 79.4%, respectively) and Park *et al.*^[12] (85%, 100%, and 95%, respectively). This lower accuracy may be due to lower experience of our sonographer for detection of triangular cord sign.

Hepatobiliary scintigraphy with technetium-labeled iminodiacetic acid analogs can be helpful for distinguishing

Table 4 Positive and negative predictive values of various diagnostic methods in BA and INH

Diagnostic method	Positive PV for BA and negative PV for INH		Negative PV for BA and positive PV for INH	
	%	<i>n</i>	%	<i>n</i>
Clinical evaluation	50	16/32	90.9	30/33
Liver enzymes	33.3	13/39	76.9	20/26
Ultrasonography	47.6	10/21	79.5	35/44
Hepatobiliary scintigraphy	40	16/40	88	22/25
Liver biopsy	90.5	19/21	100	44/44

PV: Predictive value.

biliary atresia from neonatal hepatitis and other causes of cholestasis. The sensitivity and specificity of scintigraphy in detecting of obstruction range from 83% to 100% and 33% to 100%, respectively^[1]. In the present study, scintigraphy had sensitivity and specificity of 84.2% and 47.8% for detecting BA, respectively. Scintigraphy adds little to the routine evaluation of the cholestatic infant, but may be of value in determining patency of the biliary tract, thereby excluding BA^[1].

Hepatobiliary scintigraphy had an accuracy of 58.5% in this study that is comparable to the data reported by Park *et al.*^[12], and Gupta *et al.*^[3], but lower than that by Lin *et al.*^[11] and Nadal *et al.*^[13].

Percutaneous liver biopsy is generally employed in the evaluation of neonatal cholestasis, particularly when biliary tract obstruction is high on the differential diagnosis^[14]. The NASPGHAN guideline recommends that a percutaneous liver biopsy to be performed in most infants with undiagnosed cholestasis^[1]. The biopsy should be interpreted by a pathologist with expertise in pediatric liver disease. Biopsy is recommended before performing a surgical procedure to diagnose biliary atresia. If the results are equivocal and biopsy was performed when the infant was < 6 wk of age, a repeated biopsy may be necessary.

Liver needle biopsy is the most invasive method among the various tests, but it is also the most accurate one. Our study found that liver needle biopsy was the most reliable method with the highest accuracy rate of 96.9%, which is similar to those in previous reports^[2,12,15].

In conclusion, clinical evaluation by an experienced pediatric hepatologist and liver needle biopsy might be the most reliable methods to differentiate idiopathic neonatal hepatitis and biliary atresia.

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Effect of electroacupuncture at Sibai on the gastric myoelectric activities of denervated rats

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Abstract

AIM: To explore the mechanism of the exciting effects of electro-acupuncture (EA) at Sibai on the gastric myoelectric activities.

METHODS: A total of 32 rats were randomly divided into four groups. Through intraperitoneal injection with atropine (the anti-cholinergic agent by blockade of muscarinic receptors), hexamethonium (automatic nerve ganglion-blocking agent) and reserpine (anti-adrenergic agent by depleting the adrenergic nerve terminal of its norepinephrine store), effects of EA at Sibai on the gastric myoelectric activities of the denervated rats were observed.

RESULTS: After intraperitoneal injection of atropine and hexamethonium, the average amplitude and ratio of period to time in the phase of high activity of gastric myoelectric slow wave, and the average numbers of the peaks of gastric myoelectric fast wave were significantly decreased ($P < 0.01$, $P < 0.05$, $P < 0.01$), while after intraperitoneal injection of reserpine, the aforementioned three parameters were increased ($P < 0.01$, $P < 0.05$, $P < 0.01$). EA at Sibai point partially relieved the inhibitory effect of atropine and hexamethonium on the gastric myoelectric activities in the rats ($P < 0.05$ or $P > 0.05$).

CONCLUSION: Cholinergic and adrenergic nervous systems and autonomic nerve ganglion participate in the peripheral passage of the controlling effects of EA at Foot Yangming Channel on gastrointestinal tract.

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Key words: Electro-acupuncture; Rats; Sibai; Nerve

block; Gastric myoelectric activities

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INTRODUCTION

Electroacupuncture (EA) at certain points always exhibits marked effects (inhibitory or stimulatory) on organ activities. Our previous studies indicated that EA at the Sibai point exhibited exciting effects on gastric myoelectric activities. These stimulant actions appeared on average amplitude of high activities of slow waves, the ratio between the time-course of high activities of slow waves and cycle of slow waves, average frequency of fast waves. No apparent stimulation was observed at the control point after EA. These results suggest that there are inherent specific correlations between Channels and zang and fu organs. The aim of this study was to investigate the mechanism underlying the exciting effects of EA at Sibai on gastric myoelectric activities. To this purpose, atropine (the anti-cholinergic agent by blockade of muscarinic receptors), hexamethonium (automatic nerve ganglion- blocking agent) and reserpine (anti-adrenergic agent by depleting the adrenergic nerve terminal of its norepinephrine store) were used before EA at Sibai. Gastric myoelectric activities of the rats with nerve blockade were observed^[2-4].

MATERIALS AND METHODS

Animals and experimental design

Healthy adult rats ($n = 32$, female or male, body mass: 200-300 g) were randomly divided into four groups ($n = 8$ per group): (1) EA at Sibai plus saline group: EA at Sibai for 30 min and simultaneously recorded gastric myoelectric activities for 60 min as baseline (control); EA repetition and recording after intraperitoneal injection (ip) with 2 mL saline. (2) EA at Sibai plus atropine (ip) group: EA and recording gastric myoelectric activities same as the first group. (3) EA at Sibai plus hexamethonium (15 mg/kg, ip) group: EA and recording gastric myoelectric activities same as the first group. (4) EA at Sibai plus reserpine (0.5 mg/kg, ip 24 h and 48 h prior to EA) group: EA and

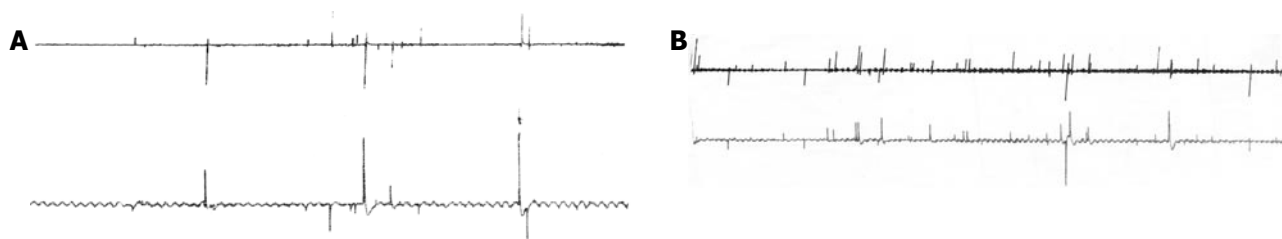


Figure 1 Slow and fast wave of the rats' gastric myoelectric activities before and after EA at Sibai. **A:** Before EA; **B:** After EA.

recording gastric myoelectric activities same as the first group.

Acupuncture points location and parameters for EA stimulation

The acupuncture points were chosen and located according to Lin's "Experimental Acupuncture" - "diagram of acupuncture points for experimental animals"^[1]. Comparative anatomic methods were also used as reference to locate the acupuncture points on rat. Parameters for EA stimulation, namely slow and fast waves (slow wave: 4 Hz; fast wave: 50 Hz), impulse width 0.5 ms, output voltage 2-4 V, output electricity 4-6 mA, 0-60 peak altitude (1 K Ω loaded), slight shake of the needle, were determined as threshold intensity. Stimulation time 30 min and output stimulation intensity were adjusted every 10 min interval to maintain proper intensity.

Placement of gastric electrode

Rats were deprived of food but not water 24 h prior to experiments. Rats were weighed and anesthetized with 10% urathe 0.5 mL (100 mg, ip), tightened and faced up. Abdomen was opened along the middle line. One platinode (diameter 2 mm) was placed under the gastric sinus portion and approximately 0.5 cm to the pyloric sphincter. The other platinode was placed on the body of the stomach and close to the first platinode, 1 cm. The cables were leaded to the skin and tightened with a plastic tube around the neck. Penicillin was injected to prevent infection. The experiments were processed after 7 d.

Recording of gastric myoelectric activities

Rats were deprived of food but not water 24 h prior to experiments. Rats were weighed and anesthetized with 10% urathe 0.5 mL (100 mg ip). Two cables were connected with the front amplifier of the two channels of the physiological recorder. Fast wave and slow wave were recorded simultaneously. Recording parameters for fast wave were: time constant 0.02 s, high frequency filter 30 Hz, and sensitivity 1 mV/cm. Recording parameters for the slow wave were: time constant 2 s, filtration 10 Hz, sensitivity 5 mV/cm. Paper speed was 10 mm/min.

Measurement of fast wave and slow waves of gastric myoelectric activities

Three minutes of wave shape, regularity, and a maximum of wave width of the slow wave were defined as one section; four such sections were accumulated and calculated the average amplitude of one section. Cycle

of slow wave was chosen in at least 3 sections. The cycle period and time courses of the high activity phase were measured, and the ratio of these two measurements were calculated. Consecutive 10 min of all fast waves were defined as one section; three such sections were accumulated and calculated the average frequency (time/min) of one section. The difference between these two measurements which treated before and after was also calculated.

Statistical analysis

Data were expressed as mean \pm SD. Paired *t* test was used to compare the difference before and after treatment within the same group. The difference among groups was analyzed by one-way ANOVA or *q* test. All the data were processed with SPSS11.5 software.

RESULTS

Effect of EA on average amplitude of high activity phase of slow wave in anesthetized rats

There was no significant difference in average amplitude of high activity phase of slow wave among groups after EA. Both atropine and hexamethonium attenuated the average amplitude of high activity phase of slow wave ($P < 0.01$ *vs* before blocking agent; $P < 0.01$ *vs* Sibai plus saline group) (Figure 1). EA markedly abolished the inhibition induced by both atropine and hexamethonium and restrained the activity ($P < 0.05$ *vs* blocking agents alone). Reserpine obviously increased the average amplitude of high activity phase of slow wave ($P < 0.01$ *vs* before blocking agent; $P < 0.01$ *vs* Sibai plus saline group). EA at Sibai still enhanced the average amplitude even after pretreatment with reserpine ($P < 0.05$ *vs* (1) after EA) (Table 1, Figure 1).

Effect of EA on the ratio between time courses of high activity of slow wave and slow wave cycle in anesthetized rats

As shown in Table 2, there was no marked difference in the ratio between time courses of high activity of slow wave and slow wave cycles in anesthetized rats. Both atropine and hexamethonium decreased the ratio ($P < 0.05$ *vs* (1) after EA). EA restrained the inhibition and recovered the ratio to the prior value before using blocking agent ($P > 0.05$). Reserpine slightly increased the ratio without significant difference compared to the value before using blocking agent. EA at Sibai after reserpine application markedly enhanced the ratio ($P < 0.05$) (Table 2).

Table 1 Effect of EA on amplitude of high activity phase of slow wave in rats (mean \pm SD, mV)

Group	(1) After EA	(2) Blocking agent	(3) Blocking agent + EA	(2)-(1)	(3)-(1)
Sibai + saline	5.35 \pm 0.32	5.21 \pm 0.76	5.38 \pm 0.26	0.14 \pm 0.32	0.03 \pm 0.09
Sibai + atropine	5.31 \pm 0.44	2.38 \pm 0.71 ^b	5.21 \pm 0.54 ^a	2.93 \pm 0.35 ^d	0.10 \pm 0.11
Sibai + hexamethonium	5.28 \pm 0.48	2.65 \pm 0.66 ^b	5.16 \pm 0.62 ^a	2.63 \pm 0.38 ^d	0.12 \pm 0.10
Sibai + reserpine	5.34 \pm 0.62	5.55 \pm 0.52 ^a	5.61 \pm 0.57 ^a	0.59 \pm 0.21 ^c	0.27 \pm 0.22 ^c

^a $P < 0.05$, ^b $P < 0.01$ vs (1) after EA; ^c $P < 0.05$, ^d $P < 0.01$ vs Sibai plus saline group.

Table 2 Effect of EA on the ratio between time courses of slow wave and slow wave cycle in rats (mean \pm SD, mV)

Group	(1) After EA	(2) Blocking agent	(3) Blocking agent + EA	(2)-(1)	(3)-(1)
Sibai + saline	0.49 \pm 0.04	0.48 \pm 0.04	0.48 \pm 0.04	0.01 \pm 0.01	0.00 \pm 0.01
Sibai + atropine	0.47 \pm 0.05	0.46 \pm 0.04 ^a	0.48 \pm 0.05	0.01 \pm 0.01	0.01 \pm 0.01
Sibai + hexamethonium	0.48 \pm 0.06	0.47 \pm 0.04 ^a	0.48 \pm 0.06	0.01 \pm 0.01	0.01 \pm 0.01
Sibai + reserpine	0.48 \pm 0.06	0.49 \pm 0.06	0.49 \pm 0.05 ^a	0.01 \pm 0.01 ^c	0.01 \pm 0.01

^a $P < 0.05$ vs (1) after EA; ^c $P < 0.05$ vs Sibai plus saline group.

Table 3 Effect of electroacupuncture (EA) on average frequency of fast wave in anesthetized rats (mean \pm SD, mV)

Group	(1) After EA	(2) Blocking agent	(3) Blocking agent + EA	(2)-(1)	(3)-(1)
Sibai + saline	36.90 \pm 10.95	36.11 \pm 9.55	36.32 \pm 9.69	0.79 \pm 0.98	0.58 \pm 0.92
Sibai + atropine	36.38 \pm 11.77	34.33 \pm 10.63 ^b	36.08 \pm 11.32	2.05 \pm 0.99 ^c	0.30 \pm 0.88
Sibai + hexamethonium	37.04 \pm 11.08	35.21 \pm 10.23 ^b	36.65 \pm 11.12	1.83 \pm 0.90 ^c	0.39 \pm 0.94
Sibai + reserpine	37.34 \pm 9.66	38.24 \pm 10.14 ^a	38.33 \pm 11.13 ^a	0.90 \pm 0.92	1.01 \pm 0.90

^a $P < 0.05$, ^b $P < 0.01$ vs (1) after EA; ^c $P < 0.05$ vs Sibai plus saline group.

Effect of EA on average frequency of fast wave in anesthetized rats

No significant change in the average frequency of fast wave was observed among groups after EA practice. Atropine and hexamethonium significantly reduced the average frequency of fast wave ($P < 0.01$ vs (1) after EA). The decreased average frequency induced by atropine or hexamethonium also appeared different with the saline group ($P < 0.05$). EA at Sibai slightly increased average frequency of fast wave, but did not reach the significance. Rats pretreated with reserpine exhibited increased average frequency ($P < 0.05$ vs (1) after EA) (Table 3).

DISCUSSION

“Twelve channels connect with arms and body surface outwardly and associate with zang and fu organs inwardly”. This theory has been widely recognized in channel theory and zang/xiang quotation of acupuncture, one of the branches of traditional Chinese medicine. The points on the body surface deeply correlate with zang and fu organs. “Following channels to select points” - this theory has been adapted in clinical practices. Experiments and clinical practices indicate that electroacupuncture at some points exhibits significant effects on certain organs. The subsequent challenge is how to clarify the mechanism underlying the correlation between points and organ activities. Nerve system and endocrine system dominate

most of the organ function and activities. The role of the nerve system in gastric myoelectric activities induced by electroacupuncture at Sibai is the main topic in this study.

Atropine is a typical anti-cholinergic agent, which acts through blockade of muscarinic receptors. Hexamethonium is a ganglion-blocking agent. Reserpine has been used to address the role of adrenergic nerve in physiological study; and it exhibits anti-adrenergic effect by depleting norepinephrine storage in the terminals. For this study, atropine, hexamethonium, and reserpine were administered by intraperitoneal injection to rats. Our results showed that both atropine and hexamethonium abolished the increased gastric activities induced by EA. The gastric activities were inhibited after both atropine and hexamethonium pretreatment. Average amplitude of high activity phase of slow wave, the ratio between time-courses of high activity phase of slow wave and slow wave cycle, and average wave frequency of fast wave were significantly reduced. These results suggest that cholinergic and sympathetic nerves are involved in the regulation induced by EA at point of Foot Yangming Channel. EA at Sibai exhibited strong exciting effects and antagonized the inhibition induced by both atropine and hexamethonium. These effects included enhanced average amplitude of high activity phase of slow wave, the ratio between time-courses of high activity phase of slow wave and slow wave cycle, and average wave frequency of fast wave. Thus, we can draw an important conclusion that both postganglionic

cholinergic nerve and preganglionic sympathetic nerve are involved in the gastric exciting effects induced by EA at Sibai. Meanwhile, both atropine and hexamethonium only partially inhibited the gastric activities. These experiments suggested that a third “no cholinergic, no adrenergic” nerve fiber may participate to control gastric myoelectric activities. Peptide nerve may mediate the gastric myoelectric activities. EA at Sibai may encourage some peptide transmitters release and regulate gastric activities. It has been reported that peptides are related to gastrointestinal mobility^[5,6,8]. EA at Zusanli, Tiansu, and Liangmeng of Foot Yangming Channel increased the complex mobility of gastric sinus portion, small intestine and closed empty intestine. Motilin, gastrin and substance P concentrations were increased. Acupuncture can incompletely abolish the inhibitory action on gastric motility induced by atropine^[7,9,10]. EA at Zusanli point of Foot Yangming Channel exhibits exciting effects on gastric myoelectric activities (amplitude, time-course, and frequency of wave) in anesthetized rats. Intravenous injection with atropine inhibits these effects^[11-14]. Contrast with this result, EA at Zusanli exhibits opposing effects on gastric myoelectric activities in awaken rabbits: inhibition of gastric mobility, gastric myoelectric frequency, and wave amplitude^[15,16]. Some authors suggest that the inhibition induced by EA at Zusanli on gastric activities is not inhibitory. The baseline control of gastric myoelectric activities plays an important role in the regulation of EA^[14,17,18]. EA leads to exciting effects for low gastric activities and inhibitory effects for high gastric activities. This phenomenon has been recognized as “Body-Organ” reflection^[19-21]. Most experiments proved that nerve-body fluid factors are involved in the effects of EA at points of Foot Yangming on gastric mobility function. Related experiments indicate that the inhibition of gastric mobility can be restrained by acupuncture at different points (head, body, and lower limb) of Foot Yangming Channel. Substance P, motilin, gastrin, and growth-inhibitory factors in gastric sinus section and medulla oblongata vary correspondingly. Acupuncture at Foot Yangming Channel regulates gastric mobility possibly through activating and releasing brain intestinal peptide (BIP) from central and local areas^[22-24]. Atropine reduces gastric myoelectric activities and BIP release. Acupuncture at Zusanli partially relieves the inhibitory effect and BIP content. These results indicated that peptide nerve may be involved in the efferent portion of acupuncture. Motilin, gastrin and substance P *etc.* released from peptide nerve participated in the process in which EA restored the inhibited gastric myoelectric activities to normal properties^[25-27], which is consistent with our present study.

Reserpine is an adrenergic nerve-blocking agent, which acts by depleting the adrenergic nerve terminal of its norepinephrine and 5-HT transmitters store^[28,29]. The gastric mobility was enhanced after the intraperitoneal injection of reserpine, suggesting that adrenergic nerve is involved in the gastric myoelectric activities regulation. Catecholamine participates in this process^[30]. Effects of acupuncture on gastric activities are related to domination of the vagus nerve and other exciting nerves on the basis of denervation of adrenergic action.

In conclusion, cholinergic and adrenergic nervous systems and autonomic nerve ganglion participate in the peripheral passage of the controlling effects of EA at the Foot Yangming Channel on gastrointestinal tract.

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CASE REPORT

Endoscopic placement of flatus tube using "lasso" technique with snare wire

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Abstract

A 55-year old man presented with acute sigmoid volvulus. The distal level of obstruction was above the level which could be reached by the rigid sigmoidoscope to allow decompression, and so a flatus tube was "lassoed" onto the side of a flexible endoscope which allowed accurate placement under direct vision. This technique allows accurate placement of catheters, feeding tubes and other devices endoscopically, which cannot be placed through the instrument channel of the endoscope.

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Key words: Endoscopy; Sigmoid volvulus; Flatus tube

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INTRODUCTION

Acute sigmoid volvulus is frequently encountered in clinical practice. The distal level of obstruction can be reached by the rigid sigmoidoscope to allow decompression. A flatus tube (Figure 1) can be "lassoed" onto the side of a flexible endoscope which allows accurate placement under direct vision. This technique allows accurate placement of catheters, feeding tubes and other devices endoscopically, which cannot be placed through the instrument channel of the endoscope.

CASE REPORT

A 55-years old male presented acutely to the surgical on-



Figure 1 Flatus tube used in our study.

call team at our institution with a 2-d history of absolute constipation and abdominal distension followed by vomiting. He had a previous history of laryngotomy for carcinoma and hypertension.

Clinical examination revealed abdominal distension, obstructive bowel sounds and an empty rectum. Plain abdominal radiology demonstrated features of distal large bowel obstruction and computed tomography revealed colonic distension to the level of the sigmoid colon, indicating a sigmoid volvulus and decompression was planned.

Under intravenous sedation with propofol, the colonoscope was passed into the sigmoid colon and the proximal colon was decompressed, but on removal of the endoscope, the sigmoid colon re-volved above the proximal extent of the rigid sigmoidoscope.

We employed a novel method for placement of the flatus tube under direct vision by 'lassoing' an endoscopic snare wire (passed through the colonoscope) onto the end of the flatus tube. The free end of the flatus tube was clamped to prevent escape of insufflated air during placement. The colonoscope (with 'lassoed' flatus tube) was renegotiated into the sigmoid colon and once satisfactory positioning was achieved under direct vision, the snare was loosened and slipped over the distal end of the flatus tube, which was left in place after the scope was withdrawn. The flatus tube was unclamped and left *in-situ* with a drainage bag attached for 48 h. The patient was allowed full diet after the procedure and discharged on the third day.

DISCUSSION

Acute sigmoid volvulus is frequently encountered in clinical practice. However, no previous report has described

use of an endoscopic snare to guide the placement of either a hollow tube (to allow drainage or installation of substances) or a measuring catheter under direct vision. We employed a novel method for placement of the flatus tube under direct vision by 'lassoing' an endoscopic snare wire (passed through the colonoscope) onto the end of

the flatus tube. The patient was allowed full diet after the procedure and discharged on the third day. It is suggested that this technique can achieve rather good results in the treatment of acute sigmoid volvulus. This technique can also be employed for upper alimentary procedures such as the accurate placement of feeding tubes or pH catheters.

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CASE REPORT

Autoimmune pancreatitis associated with a large pancreatic pseudocyst

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Abstract

Pancreatic cystic lesions comprise various entities with different histopathological characteristics and their differential diagnosis is often a challenge for clinicians. Autoimmune pancreatitis (AIP) is usually not considered in the differential diagnosis of cystic lesions, but often mimics the morphological aspects of pancreatic neoplasm. We report the case of a 64-year-old male patient with a cystic pancreatic head lesion (diameter 5 cm) and stenosis of the distal bile duct requiring repeated stenting. Because of the clinical presentation together with moderate elevation of serum CA19-9 and massive elevation of cyst fluid CA19-9 (122.695 U/L; normal range: < 37.0 U/L), the patient underwent explorative laparotomy and pylorus preserving partial pancreaticoduodenectomy. Histology revealed surprisingly AIP with an inflammatory pseudocyst. In conclusion, cyst fluid analysis of tumor markers and cyst fluid cytology lack high accuracy to clearly differentiate cystic pancreatic lesions. Although AIP is rarely associated with pseudocysts, the disease has to be considered in the differential diagnosis of cystic pancreatic lesions. Early examination of serum IgG, IgG₄ and auto-antibodies might save these patients from unnecessary endoscopical and surgical procedures.

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Key words: Pseudocyst; Autoimmune pancreatitis; Pancreatic cancer; Tumor marker; CEA; CA19-9

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INTRODUCTION

Autoimmune pancreatitis (AIP) is a benign disease that responds well to steroid treatment. Characteristics include radiological evidence of an irregular narrowing of the pancreatic main duct and a diffuse enlargement of the pancreas, together with increased levels of serum IgG and the IgG₄ subclass as well as antinuclear (ANA), antilactoferrin (ALF), anticarbonic anhydrase II antibodies (ACA-II) and rheumatoid factor^[1]. On histological examination, periductal lymphoplasmacytic infiltration, periductal fibrosis and venulitis are most frequently observed^[2]. Although there is no international consensus on the diagnostic criteria for AIP, histology should be considered the gold standard in cases in which tissue diagnosis is possible. AIP predominantly affects male patients and is often associated with other autoimmune diseases such as Sjögren's syndrome, primary sclerosing cholangitis or diabetes mellitus which lead to the hypothesis that AIP might be part of a systemic autoimmune disorder with increased IgG₄ and immune complexes (secondary AIP)^[1]. Although AIP has become more and more accepted as a distinct disease entity during the last years^[3], the presentation of the disease can be unusual and misleading as depicted in the reported case. The correct diagnosis of AIP is of great clinical relevance since this pancreatic disorder, in contrast to most other pancreatic pathologies, can be treated successfully by non-invasive therapies.

CASE REPORT

A 64-year-old male patient was referred to our department with a cystic lesion of the pancreatic head and a history of multiple biliary stent placements during a course of two years due to distal bile duct stenosis. There was no history of alcohol abuse. Three years earlier, the patient had undergone an explorative laparotomy, cholecystectomy and lymph node biopsy at a different hospital because of a pancreatic tumor of unknown etiology. However, intraoperative assessment and histology did not reveal neoplastic tumor growth. On follow-up, the patient developed recurrent attacks of abdominal pain with mild to moderate pancreatic enzyme elevation, and a distal bile duct stenosis requiring repeated stenting and dilation (Figure 1A). Fine needle aspiration cytology of the pancreas was consistent with chronic pancreatitis. On further follow-up, a cyst of the pancreatic head (diameter 5 cm) was diagnosed (Figures



Figure 1 MRCP (A) depicting the dilated bile duct (13 mm) in close vicinity to the pancreatic cyst; CT scans (B, C) showing the cystic pancreatic lesion in the pancreatic head (arrows).

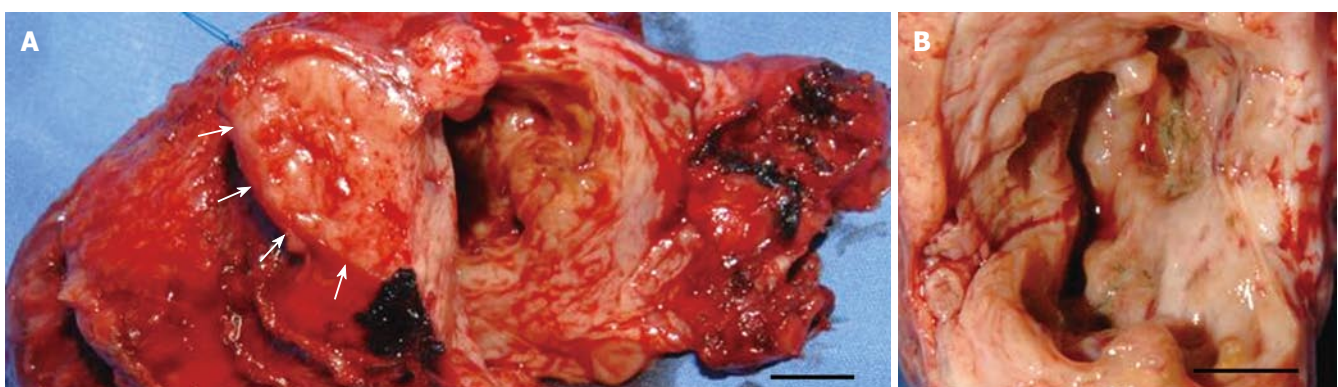


Figure 2 Intraoperative findings of the resected pancreatic specimen. A, B: Macroscopic appearance of the pancreaticoduodenectomy specimen (A) and the opened pseudocyst (B). Arrows indicate the pancreatic cut margin. Scale bar: 1 cm.

1B and C). Cyst fluid analysis showed a CEA of 55 ng/L (normal range: < 3.4 ng/L) and a CA19-9 of 122.695 U/L (normal range: < 37.0 U/L), without evidence of malignant cells. The patient was referred to our department for surgical therapy with the suspicion of a cystic neoplasm. Pertinent laboratory data on admission to our department were (in brackets: normal range): lipase 108 U/L (< 51 U/L), γ -glutamyl transferase 158 U/L (< 60 U/L), bilirubin 0.4 mg/dL (within normal range), calcium 2.32 mmol/L (within normal range), WBC 6.91/nL (within normal range), CA19-9 43.4 U/mL (< 37.0 U/mL), CEA 3.2 ng/mL (< 2.5 ng/mL).

The patient underwent a pylorus preserving partial pancreaticoduodenectomy. The postoperative course was uneventful and the patient was discharged after seven days. Grossly, the pancreatic head measured 7 cm \times 5 cm \times 3 cm and showed a whitish induration of the tissue with a cyst of 4 cm in diameter (Figure 2A and B). Histological examination revealed surprisingly autoimmune pancreatitis with an inflammatory pseudocyst without evidence for malignancy (Figure 3A and F).

DISCUSSION

Differentiating cystic pancreatic lesions remains a clinical challenge^[4]. Adding serological analysis (e.g. CEA, CA19-9) of aspirated cyst fluid to conventional imaging modalities

results in additional accuracy. Thus, of various markers including tumor markers and cytology, a CEA cutoff of 192 ng/mL (in the cyst fluid) demonstrated the highest accuracy in differentiating mucinous from non-mucinous cystic lesions^[5]. However, the sensitivity and specificity rates remain unsatisfactory. Therefore, resection of cystic lesions remains the treatment of choice if malignant or pre-malignant cystic neoplasm cannot be ruled out^[6].

AIP, on the other hand, is rarely associated with pseudocysts^[7,8], but involvement of the pancreatic head can mimic pancreatic neoplasm^[3]. AIP responds well to steroid treatment and is often associated with elevated serum IgG/IgG₄ and the presence of different auto-antibodies (e.g. ANA, and others)^[3,9]. In addition, pseudocysts in patients with AIP might represent a highly active inflammatory process, and these lesions have been shown to be steroid responsive if associated with AIP^[7]. In the present case, histology demonstrated that the inflammatory process involved the distal common bile duct, which is most likely the reason for the patients' history of recurrent bile duct stenosis.

The pseudocyst formation was detected almost 2 years after onset of abdominal symptoms. Muraki *et al* observed pancreatic cysts 1-3 years after onset of abdominal symptoms or diagnosis of AIP^[7], indicating that pseudocyst development in AIP occurs after prolonged disease activity.

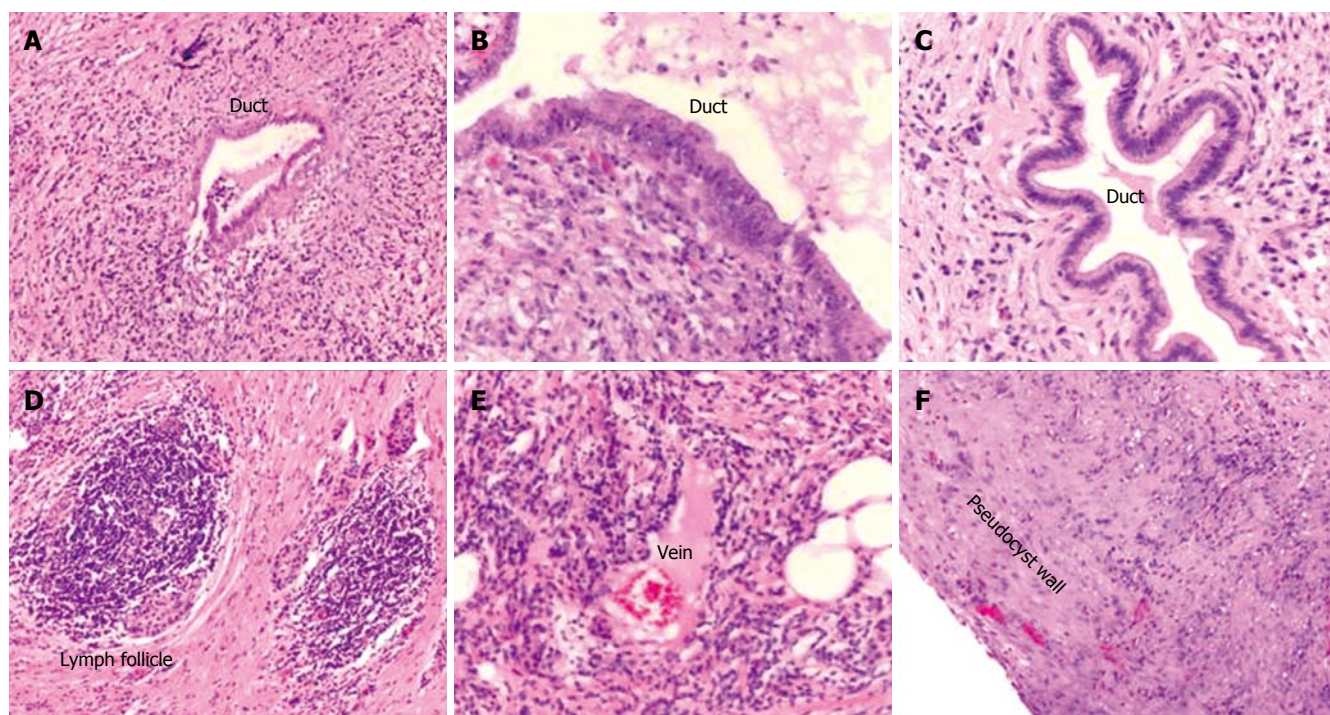


Figure 3 Histological findings of the resected pancreatic specimen. Histological examination displaying marked chronic periductal lymphoplasmacytic inflammation and fibrosis (A-C), intrapancreatic lymph follicle (D), and venulitis (E). F depicts the wall of the pseudocyst without evidence of epithelial lining.

In our case, the consideration of AIP before or after the first surgical exploration together with a trial of steroid therapy would likely have saved the patient a number of endoscopic and ultimately surgical therapies. This case highlights the clinical problem of differentiating cystic pancreatic lesions, and the importance of considering AIP as a differential diagnosis early in the course of the disease. Nonetheless, given the high cure rate of pancreatic cystic tumors with surgical resection, laparotomy should not be delayed in case of suspected malignancy.

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CASE REPORT

Is acute dyspnea related to oxaliplatin administration?

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Abstract

The standard adjuvant treatment of colon cancer is fluorouracil plus leucovorin. Oxaliplatin improves the efficacy of this combination in patients with stage III colon cancer and moreover its toxicity is well tolerable. We describe a rare clinical case of acute dyspnoea probably related to oxaliplatin at one month from the end of the adjuvant treatment. A 74-year-old man developed a locally advanced sigmoid carcinoma (pT3N1M0). A port a cath attached to an open-ended catheter was implanted in order to administer primary chemotherapy safely according to the FOLFOX4 schedule. One month following the end of the 6th cycle, the patient referred a persistent cough and moderate dyspnoea. Chest radiography displayed a change in the lung interstitium, chest CT scan confirmed this aspect of adult respiratory distress syndrome, spirometry reported a decreased carbon monoxide diffusion capacity. Antibiotic and corticosteroids were administered for 10 d, then a repeated chest X ray evidenced a progressive pulmonary infiltration. A transbronchial biopsy and cytology did not show an infective process, a CT scan reported radiological abnormalities including linear and nodular densities which were becoming confluent. Antimicrobial and antiviral drugs did not evidence any benefit. The antiviral therapy was stopped and high dose methylprednisolone was started. The patient died of pulmonary distress after 10 d.

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Key words: Acute dyspnea; Oxaliplatin; Colon cancer

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INTRODUCTION

For almost 15 years, adjuvant chemotherapy has been known to improve disease-free survival (DFS) and overall survival (OS) in colon cancer patients. The pivotal study of Moertel *et al* in 1990^[1], demonstrated that OS and DFS are improved after 12 mo of treatment with bolus 5-fluorouracil (5-FU) and levamisole, which has led to the First National Cancer Institute (NCI) consensus recommendation for stage III colon cancer^[2]. Subsequent studies conducted in the 1990s have established 6 to 8 mo of adjuvant therapy with bolus 5-FU plus leucovorin (LV) as standard of care. The positive results of the international multi-center study of oxaliplatin/5-fluorouracil/leucovorin in the adjuvant treatment of colon cancer (MOSAIC) trial^[3] enrolling both stage II and III patients, have led to US Food and Drug Administration approval of oxaliplatin plus 5-FU/LV (FOLFOX4) for patients with stage III colon cancer in November 2004^[4], which followed the European approval as adjuvant treatment of stage III (Dukes C) colon cancer after complete resection of the primary tumour in September 2004. The US Food and Drug Administration approval is based on the demonstration of the statistical superiority of FOLFOX4 to infusional plus bolus 5-FU/LV (LV5FU2 regimen) on 3- and 4-year DFS in the stage III subgroup of patients in the MOSAIC trial^[3-5]. Vomiting is observed in about 47% of cases, granulocytopenia in about 79% of cases and thrombocytopenia in 77% of cases, paresthesia in about 92% of cases and increased enzymes in about 57%, respectively. Other types of toxicities are of low grade.

CASE REPORT

We describe a rare clinical case of acute dyspnoea at one month from the end of adjuvant treatment.

A 74-year old man developed a locally advanced sigmoid carcinoma (pT3N1M0). A port a cath attached to an open-ended catheter, was implanted in order to administer primary chemotherapy safely according to the FOLFOX4 schedule (oxaliplatin 85 mg/m² d 1, 5-FU 400 mg/m² d 1, 2 administered as bolus, 5-FU 600 mg/m² d 1, 2 administered by 22 h continuous infusion and LV 100 mg/m² administered by 2 h infusion, every 2 wk). The 6-mo administration went without acute complications (except for a grade 1 thrombocytopenia and neutropenia). One month following the end of the 6th cycle, the patient

referred a persistent cough and moderate dyspnoea. Chest radiography documented a change in the lung interstitium, chest CT scan confirmed this aspect of adult respiratory distress syndrome, spirometry reported a decreased carbon monoxide diffusion capacity. Antibiotic and corticosteroids were administered for 10 d; a repeated chest X ray evidenced a progressive pulmonary infiltration. The patient was urgently admitted to our hospital for increasing fever and dyspnoea. Transbronchial biopsy and cytology did not show an infective process, CT scan reported radiological abnormalities including linear and nodular densities which were becoming confluent. Antimicrobial and antiviral drugs did not evidence any benefit. For a respiratory complication the patient was admitted to the Intensive Care Unit. The antiviral therapy was stopped and high dose methylprednisolone was started. The patient died of pulmonary distress after 10 d.

DISCUSSION

To our knowledge, this is one of the very few reports on such a phenomenon in patients with colorectal cancer (CRC) during FOLFOX4 chemotherapy. Rare cases of acute interstitial lung disease and of pulmonary fibrosis have been reported after oxaliplatin, including obliterated bronchiolitis with organized pneumopathy or interstitial pneumonia-like lung disease. The MOSAIC adjuvant trial has not reported such toxicity^[3].

In our clinical case, the interstitial aspect of lung fibrosis appeared to be rapidly evolutionary, showing no

improvement 7 d after steroid therapy.

Since the use of oxaliplatin chemotherapy has increased dramatically in the past 2 years in CRC patients, and because of the lack of clinical data in literature, we strongly recommend accurate basal pulmonary analysis with spirometry, especially in elderly patients, to evaluate the respiratory reserve and careful monitoring of any respiratory distress which may occur in patients during or at the end of oxaliplatin chemotherapy. Clinicians should be aware of the potential of lung toxicity caused by novel antineoplastic agents.

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CASE REPORT

Removal of press-through-packs impacted in the upper esophagus using an overtube

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Abstract

Foreign bodies in the upper esophagus should be removed as soon as possible to avoid serious complications. However, removals of foreign bodies in the upper esophagus are very difficult, especially if they have sharp edges, such as press-through-packs (PTPs). We experienced four cases of the impacted PTPs in the upper esophagus which was successfully extracted endoscopically with the overtube. Because two edges of PTPs were so firmly impacted in the esophageal wall in all cases, the PTPs were not movable in the upper esophagus. However, after insertion of the overtube, PTPs became movable and were successfully extracted and no serious complications occurred after extraction of PTPs. In one case, insertion of the overtube rapidly expanded the upper esophagus and PTP progressed to the gastric cavity and it could be extracted with the endoscopic protector hood. The endoscopic removal with the overtube was a simple, safe and effective technique for the removal of the impacted PTPs in upper esophagus.

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Key words: Esophagus; Foreign body; Endoscopy

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INTRODUCTION

Foreign bodies in the esophagus should be removed to

avoid serious complications, such as bleeding, mediastinitis, esophageal perforation and pulmonary aspiration^[1-5]. Esophageal impaction of a foreign body with sharp edges is considered a medical emergency because it is often associated with perforation or hemorrhage, the likelihood of which increases with the passage of time^[6-8]. Therefore, such objects should be removed as soon as possible after ingestion^[9]. However, the removal of foreign bodies with sharp edges that are located in the upper esophagus is difficult. Furthermore, complications, such as mediastinitis, pneumothorax and aorto-esophageal fistula, can develop during or after removal of a foreign body from the esophagus^[10-12]. The absence of a serosal layer in the esophageal wall increases the potential for serious complications^[13].

Press-through-packs (PTPs) are widely used in Korea for packaging drugs, and cases of impaction of PTPs in the esophagus have been reported^[14,15]. Because PTPs have three or four edges that may be razor-sharp, esophageal perforation or bleeding could develop during their removal. We experienced four cases of the impacted PTPs in the upper esophagus, which were successfully extracted endoscopically with the overtube.

CASE REPORT

Four patients who accidentally swallowed PTPs visited the Emergency Room of the Korea University Guro Hospital from October 2004 to December 2005 (Table 1). The median age of the four patients was 51 years (range, 34-76 years). All patients complained of severe dysphagia. They had upper respiratory tract infections and had taken several pills such as antibiotics, analgesics, etc., one of which was contained in a PTP. The patients had no history of dementia or psychological disease. One patient (case 4) had hypothyroidism, but the others had no comorbidities. Physical examinations revealed smooth breathing sounds, soft abdomens that were not tender, and active bowel sounds. Chest and neck radiograms were unrevealing.

After intravenous injection of 25 mg of pethidine for sedation, urgent endoscopic examinations were performed on each patient using a conventional Olympus video endoscope (Olympus Optical Co. Ltd., Tokyo, Japan), which revealed a PTP impacted in the upper esophagus (Figure 1). Attempts to extract the PTPs using forceps were unsuccessful because the edges of the PTPs were firmly impacted in the esophageal walls.

After the tip of the endoscope had been placed close

Table 1 Baseline characteristics of 4 cases with impacted press-through-packs in the upper esophagus

	Sex	Age	Duration from swallowing of PTP to endoscopy (h)	Distance of impacted PTP from incisor (cm)	Size of impacted PTP (length × width; mm)
Case 1	F	50	4	18	33 × 20
Case 2	F	35	1	19	25 × 17
Case 3	M	78	3	20	31 × 21
Case 4	F	52	8	19	23 × 21

**Figure 1** Endoscopic image showing a press-through-pack impacted in the upper esophageal wall.

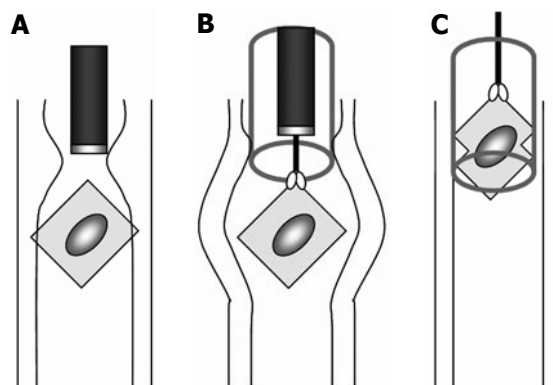
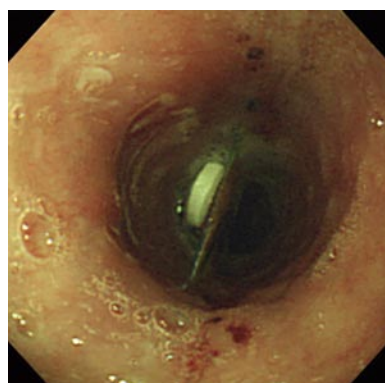
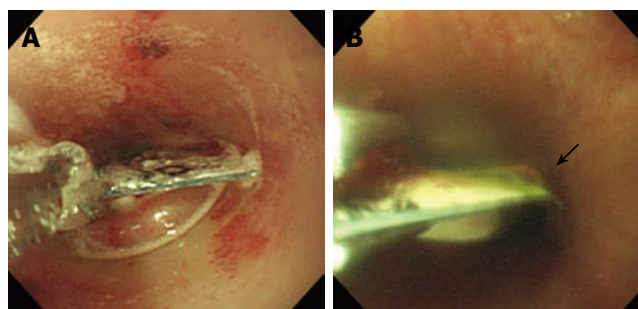
to the PTP, a flexible overtube (length, 195 mm; inside diameter, 15 mm; outside diameter, 18 mm) was passed over the endoscope to a position just proximal to the edge of the PTP (Figure 2). Insertion of the overtube resulted in relaxation of the upper esophagus, which enabled the impacted PTP to be moved (Figure 3). We used endoscopic forceps to grasp the PTP and pull it into the overtube (Figure 4). After confirming that the edges of the PTP were within the overtube, the overtube and the PTP were removed simultaneously. In all four cases, insertion of the overtube enabled movement of the PTP and successful extraction without complications, such as mediastinitis, hemorrhage, perforation, or death. After removal, erosions were visible at the sites of impactions.

In one case (case 2), insertion of the overtube expanded the upper esophagus so rapidly that the PTP progressed to the gastric cavity before it could be grasped with the forceps. After locating the PTP in the middle of the stomach lumen, we removed the overtube and extracted the PTP using the endoscopic protector hood to prevent injury to the stomach and esophagus.

After extraction of the PTPs, the patients' dysphagia improved and no other symptoms developed. The results of chest radiographies after extraction of the PTPs were unrevealing. All patients were observed overnight and discharged. Prophylactic antibiotics were not prescribed. The patients were asymptomatic during follow-up consultations one week after the procedures.

DISCUSSION

Although esophageal foreign bodies in adults usually occur in prisoners and patients with underlying esophageal diseases, mental retardation, or psychiatric illnesses^[16], esophageal impactions of PTPs frequently occur in senile

**Figure 2** Method for removal of a press-through-pack impacted in the upper esophagus. A: The press-through-pack was located using a diagnostic endoscope; B: an overtube was inserted and resulted in relaxation of the upper esophagus, which enabled the impacted press-through-pack to be moved; C: forceful pulling of the press-through-pack using forceps caused the edges of the PTP to bend, enabling it to enter the overtube.**Figure 3** Endoscopic image showing distal relocation of a press-through-pack that was previously impacted and immovable. Erosions proximal to the press-through-pack indicate the site at which the press-through-pack was impacted until insertion of the overtube.**Figure 4** A: Endoscopic image showing forceful retraction of the press-through-pack by a forceps; B shows how the edges of the press-through-pack were bent (arrow) during entry into the overtube.

people who are not members of these high-risk groups^[17], and the incidence thereof is increasing rapidly and in proportion to the ageing of the population^[17].

Because PTPs usually have sharp edges, care is needed to prevent esophageal or posterior pharyngeal injuries during endoscopic removal of PTPs from the upper esophagus. The endoscopic protector hood reportedly permits easy and safe removal of sharp or pointed foreign bodies^[18]. However, the foreign body must be moved to the gastric cavity to flip the protector's hood back to its original shape for withdrawal through the lower esophageal sphincter^[18]. Such devices cannot be used when the foreign body is so

firmly impacted that it cannot be moved. In our patients, the PTPs could not be moved until the overtube was inserted. Tsutsui *et al.*^[15] reported a case in which removal of a PTP from the esophagus was accomplished using two flexible endoscopes. However, this procedure requires two endoscopists and the insertion of additional equipment, which may cause more discomfort to the patient than an overtube. Recently, Jeon *et al.*^[8] used an oral side-balloon that is generally used in esophageal variceal sclerotherapy; it was attached to the distal part of an endoscope to release impacted sharp foreign bodies, including PTPs, from the esophageal wall. However, in our patients, the impacted PTPs were so proximal that we would have been unable to progress the endoscope far enough down the esophagus to permit ballooning. In addition, this method does not prevent pharyngeal mucosal injury. A soft large cap (18 mm in tip diameter, D-206 series, Olympus) may be used for removal of esophageal foreign bodies^[8]. However, it might not be able to cover all length of the PTPs and this incomplete coverage may lead to pharyngeal and upper esophageal mucosal injury.

We used overtubes to remove the impacted PTPs. Although the overtubes were placed just above the proximal tips of the PTPs, they relaxed the esophageal lumens to the extent that the impacted PTPs were movable. This may have been caused by expansion of the proximal esophageal lumen or induction of esophageal peristalsis by the overtube. Swallowing movements induced by insertion of the overtube may initiate primary peristalsis, and the presence of the overtube in the esophagus after primary peristalsis may stimulate sensory receptors in the esophagus to initiate secondary peristalsis^[19].

The overtube technique cannot be used for the removal of foreign bodies that are wider than the internal diameter of the overtube. Although the PTPs in all four of our cases were wider than the internal diameter of the overtube, their flexibility enabled them to be pulled into the overtube using forceps. However, care should be taken not to cause mucosal injury by pinching the mucosa between the PTP and the overtube. Such injury can be avoided by changing the site at which the PTP is grasped.

The use of an overtube during removal of an impacted PTP has several merits. Firstly, it causes expansion of the upper esophagus, which releases the impacted PTP. Although esophageal relaxation resulted in progression of the PTP to the gastric cavity in one case, it was easily extracted using the endoscopic protector hood. Secondly, it prevents aspiration and esophageal or pharyngeal injury^[4,9,20,21].

However, the overtube must be used with caution, especially when the esophageal foreign body causes esophageal stricture. In such cases, an overtube could cause esophageal perforation^[22-24]. Berkelhammer *et al.*^[23] reported that the use of an overtube for variceal ligation resulted in cricopharyngeal submucosal dissection with pneumomediastinum and proximal variceal bleeding. Therefore, endoscopic evaluation should be performed before passage of the overtube. In addition, because the impacted PTPs could lead serious complications, such as mediastinitis and aortic-esophageal fistula^[10,11],

careful evaluation should be performed to assess the relationship between PTPs and esophageal wall before endoscopic removal of PTPs. In our cases, chest and neck radiograms as well as physical examination did not show any sign of mediastinitis, and the location of PTPs were upper esophagus (18-20 cm from incisor), which were far from the aorta. Therefore, the possibility of serious complications was estimated to be very low and endoscopic removal for PTPs were performed.

In conclusion, the use of an overtube for endoscopic removal of impacted PTPs from the upper esophagus is simple, effective, and safe.

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S- Editor Liu Y L- Editor Kumar M E- Editor Bi L



CASE REPORT

Diffuse gastroduodenitis and pouchitis associated with ulcerative colitis

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Abstract

We experienced a very rare case of ulcerative colitis (UC) accompanied with analogous lesions in the stomach, duodenum, and ileal J-pouch. Ileal J-pouch anal anastomosis was performed on a 29-year old woman in 1996. Six years later, she was admitted again to our hospital because of epigastralgia, nausea, watery diarrhea and low fever. Based on the results of endoscopic examination, we diagnosed it as pouchitis. Moreover, on hypotonic duodenography, expansion of the duodenal bulb and the descending portion were poor. Kerckring folds disappeared with typical lead-pipe appearance. The pathogenesis of the gastric and duodenal lesion in this patient was similar to that of the colonic lesions of UC. For the gastroduodenal lesions in this patient, symptomatic remission was obtained following administration of crushed mesalazine tablets (1500 mg/d) for 14 d with continuous administration of omeprazole. Firstly we used ciprofloxacin to treat pouchitis. On the fifth day, she got a fever because of catheter infection. In the catheter culture, methicillin-resistant *Staphylococcus aureus* (MRSA) was detected. Therefore we changed ciprofloxacin to vancomycin hydrochloride (Vancomycin®). Vancomycin was very effective, and the stool frequency dramatically improved in three days. Now she continues to take mesalazine, but her condition is stable and there has been no recurrence of pouchitis.

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Key words: Ulcerative colitis; Pouchitis; Gastroduodenal lesions

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INTRODUCTION

Pouchitis after total colectomy with ileal pouch anal anastomosis can sometimes occur in patients with ulcerative colitis (UC). Despite intensive studies the cause of pouchitis remains unknown, but many contributing factors, such as faecal stasis, bacterial invasion, and faecal bile acids have been postulated. Data on the cumulative risk of pouchitis at 5 to 10 years after ileal J-pouch anal anastomosis (IPAA) in UC patients range from 30% to 50% in Western countries^[1]. On the other hand, the cumulative risk of pouchitis in Japanese UC patients is lower than that in Western countries^[2]. Gastroduodenal lesions in patients with UC are apparently rare, the stomach and duodenum are not generally considered as target organs in UC and there is no consensus regarding their significance or management. When we review the previous case reports, if anything, the number of reported cases of gastroduodenal lesions associated with UC is increasing in Japan. At this time, we experienced a very rare case of UC accompanied with analogous lesions in the stomach, duodenum, and ileal J-pouch. These findings suggest that UC is a generalized disease in which gastroduodenal changes can be regarded as one facet.

CASE REPORT

A 29-year old woman with a 3-year history of left-side colitis was referred to our hospital for 2 stage total proctocolectomy with mucosectomy, ileal J-pouch anal anastomosis in 1996. The diagnosis of UC was made on the basis of a barium enema and colonoscopic findings and confirmed by the typical histologic features of colorectal biopsies. No evidence of Crohn's disease (CD) was present at the time of surgery, and the gross and microscopic features of the colectomy specimen were those of UC. The postoperative course was uneventful, and oral prednisolone was gradually tapered off and completely stopped 2 mo after ileostomy closure. In

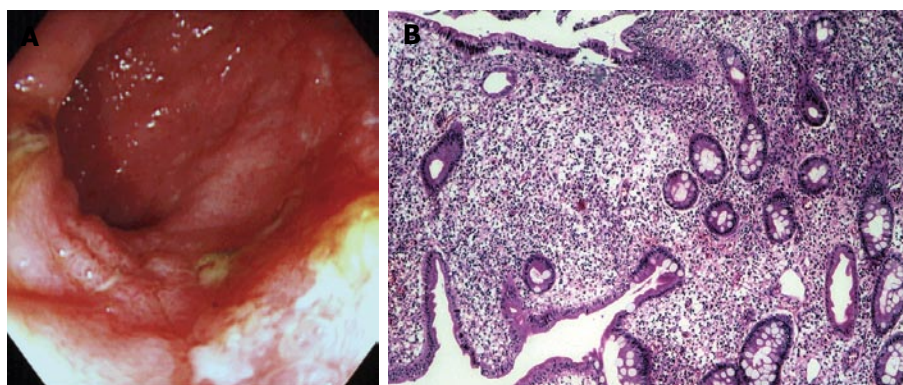


Figure 1 Endoscopy (A) and histological examination (B) showing pouchitis in the ileal J-pouch.

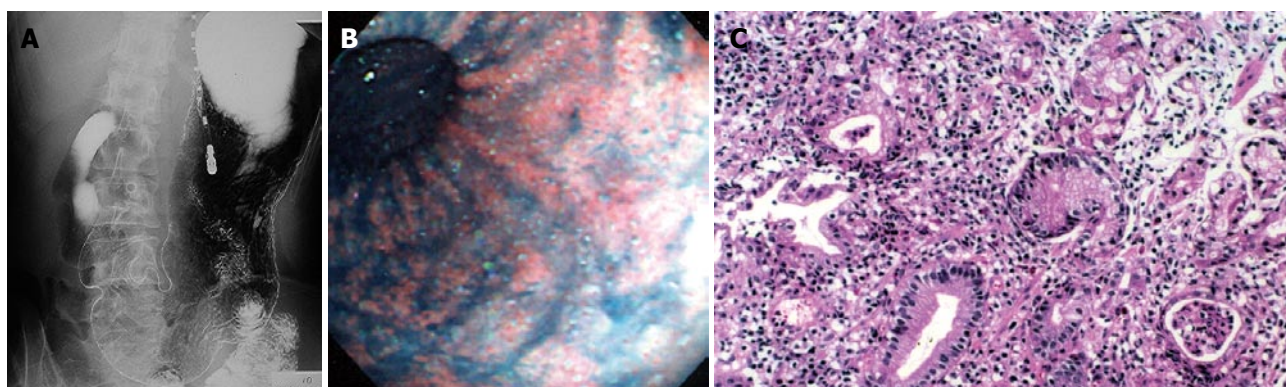


Figure 2 Upper gastrointestinal examination (A), endoscopy (B) and histological examination (C) showing lesions in the stomach.

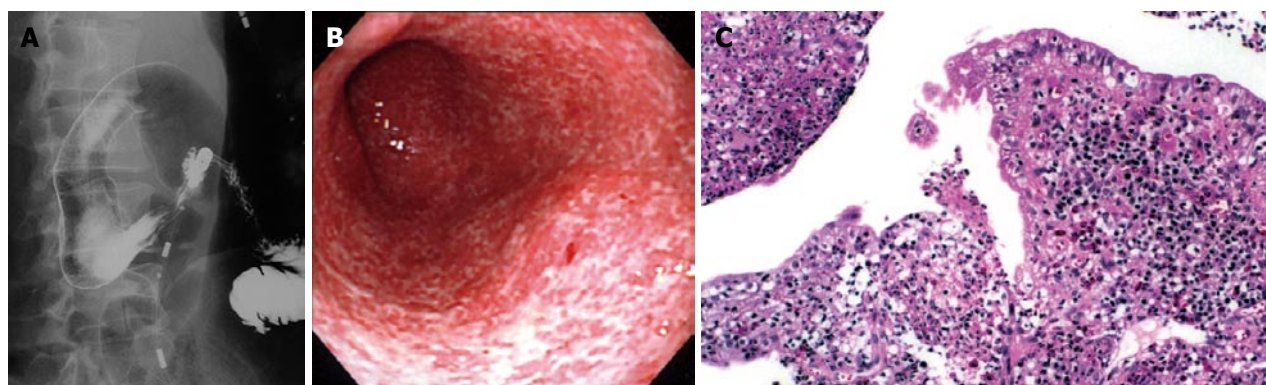


Figure 3 Hypotonic duodenography (A), endoscopy (B) and histological examination (C) showing lesions in the duodenum.

December 2002, this patient was admitted again to our hospital because of epigastralgia, nausea, watery diarrhea and low fever. On admission, the body temperature was 37.2°C and laboratory data were as follows: red blood cell count $467 \times 10^4/\mu\text{L}$, hemoglobin 12.3 g/dL, white blood cell count $6000/\mu\text{L}$. C-reactive protein (CRP) was slightly increased at 1.3 mg/dL.

At first we doubted it was pouchitis and performed endoscopy in the ileal J-pouch. The endoscopic features were edema, granularity, loss of vascular pattern, mucus exudates and ulcerations (Figure 1A). Histological examination of the ileal J-pouch biopsied in the active stage demonstrated severe infiltration of inflammatory cells in mucosa (Figure 1B). We used the pouchitis disease activity index (PDAI) as the diagnostic criteria. Her PDAI score was 12 points. We diagnosed her with pouchitis.

Endoscopy of the upper gastrointestinal tract disclosed multiple erosions and granular changes in the antral greater curvature of the stomach and similar features were noted in the duodenal bulb and the descending portion of the duodenum (Figures 2B and 3B). On hypotonic duodenography, expansion of the duodenal bulb and the descending portion were poor. Kerckring folds disappeared with a typical lead-pipe appearance (Figure 3A). The expansion of the gastric wall was kept but antral mucosa disclosed multiple granular changes (Figure 2A). Histological examination of the stomach biopsied in the active stage demonstrated severe infiltration of inflammatory cells in mucosa and crypt abscess in the mucous glandules (Figure 2C). Histology of the biopsy specimens revealed dense acute and chronic inflammatory infiltrates accompanied with cryptitis in the duodenum

(Figure 3C). Neither granuloma nor *H pylori* was detected in biopsied sections.

After admission, we performed intravenous hyperalimentation, because she exhibited dehydration due to severe watery diarrhea. The gastroduodenal lesions in this patient were not improved by oral omeprazole (20 mg/d) for 7 d, but symptomatic remission was obtained following administration of crushed mesalazine tablets (1 500 mg/d) for 14 d, with continuous administration of omeprazole. Firstly we used ciprofloxacin to treat pouchitis. On the fifth day, she got a fever because of catheter infection. Methicillin-resistant *Staphylococcus aureus* (MRSA) was detected in the catheter culture. Therefore we changed ciprofloxacin to vancomycin hydrochloride (Vancomycin®). Vancomycin was very effective, and the stool frequency dramatically improved in three days. The patient was discharged on the 44th hospital day. Now she continues to take mesalazine, but her condition is stable and there is no recurrence of pouchitis.

DISCUSSION

Pouchitis is an idiopathic inflammatory disease of the ileal reservoir in patients who have undergone ileal J-pouch anal anastomosis (IPAA), and is the most common long-term complication of IPAA in UC. The cumulative risk of developing pouchitis in Japanese UC patients is lower than that of western countries^[1,2]. On the other hand, reports of gastroduodenal lesions associated with UC are extremely rare in Western countries. In our search we found only 6 cases. Thompson and Borgen^[3] reported two cases in 1960 and Valdez *et al*^[4] reported four cases in 2000. In Japan, recently, the number of reports of gastroduodenal lesions associated with UC is increasing. In 2002, Warita *et al*^[5] reported 15 cases in Japan. The instance of both pouchitis and gastroduodenal lesion associated UC exists in only three cases. These cases were reported by Valdez *et al*^[4] in 2000. No patient with complicated pouchitis and gastroduodenal lesion has been reported in Japan.

Crohn's disease can affect the whole gastrointestinal tract. On the other hand, UC is a chronic inflammatory disease of the colon and includes various extracolonic manifestations. However, the stomach and duodenum are not generally considered target organs in UC. So in this patient, differential diagnosis from CD was important. In our patient, no evidence of CD was present at the time of surgery, and the gross and microscopic features of the colectomy specimen were those of ulcerative left-side colitis. Diffuse ulcerative duodenitis similar in appearance to the ulcerative colitis was found extending from the second to the third portion of the duodenum. Moreover, the pathogenesis of the gastric and duodenal lesions in this patient was similar to that of the colonic lesions of UC and these findings were not induced by *H pylori* infection. *H pylori* was negative in our patient, which is consistent with the finding of Warita *et al*^[5] reported who reported that five of 15 cases are negative for *H pylori* infection. Recent preliminary reports have described a reduced prevalence of *H pylori* infection in patients with inflammatory bowel disease compared with the general

population. Parente *et al*^[6] reported that previous exposure to sulphasalazine is associated with a significantly lower risk of infection, mainly in Crohn's disease but also in UC patients.

Medical treatment with mesalazine was very effective in our patient. Mitomi *et al*^[7] reported that the symptoms of their patient are resolved one month after treatment with sulfasarazopyrin (3.0 mg/d) and some antacids. Myojo *et al*^[8] reported that while the duodenitis does not respond to a proton-pump inhibitor, it improves obviously after treatment with mesalazine. Moreover, Sasaki *et al*^[9] reported that gastroduodenal lesions are not improved after oral omeprazole, but healing is obtained following intensive intravenous prednisolone therapy for 14 d with continuous administration of omeprazole. Although there is no consensus regarding these types of management, sulfasarazopyrin or mesalazine should be considered as fast-line therapies for gastroduodenal lesions. If these treatment modalities are ineffective, we should use steroids as the second-line therapy for gastroduodenal lesions.

Terashima *et al*^[10] reported that duodenal lesion accompanying UC may be a more common phenomenon, although it occurs infrequently during steroid therapy. In the future, patients with complicated gastroduodenal lesions in a postoperative steroid-free condition may increase. We have to recognize that UC is a generalized disease in which gastroduodenal changes can be regarded as one facet.

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Fukuoka, Japan
<http://www.congre.co.jp/1st-aphba>

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14th United European Gastroenterology Week, UEGW
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Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
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Falk Foundation e.V.
symposia@falkfoundation.de

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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
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veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

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Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
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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

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]

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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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- 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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Minimally invasive surgery for esophageal achalasia

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Abstract

Esophageal achalasia is the most commonly diagnosed primary esophageal motor disorder and the second most common functional esophageal disorder. Current therapy of achalasia is directed toward elimination of the outflow resistance caused by failure of the lower esophageal sphincter to relax completely upon swallowing. The advent of minimally invasive surgery has nearly replaced endoscopic pneumatic dilation as the first-line therapeutic approach. In this editorial, the rationale and the evidence supporting the use of laparoscopic Heller myotomy combined with fundoplication as a primary treatment of achalasia are reviewed.

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Key words: Esophagus; Achalasia; Laparoscopy; Heller myotomy; Gastroesophageal reflux

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INTRODUCTION

Idiopathic achalasia is a primary motor disorder characterized by incomplete relaxation of the lower esophageal sphincter and aperistalsis of the esophageal body secondary to loss of inhibitory ganglion cells in the myenteric plexus. It is unclear whether the primary event occurs in the brain or whether the neurologic changes are the result of a direct injury of the myenteric plexus. The etiology of the disease is unknown, with genetic, autoimmune, infectious, and environmental factors being implicated. Achalasia, usually diagnosed between 20 and 40 years or after 60 years of age, is the most common primary esophageal motor disorder; it is second only to gastroesophageal reflux disease as the most common

functional esophageal disorder to require surgical intervention. The first account of a successfully treated case of achalasia was described by Thomas Willis in 1674, in which a whale bone was used to forcibly dilate the cardia. The first surgical myotomy was performed by Ernst Heller in 1913.

PATHOPHYSIOLOGY AND ASSESSMENT OF ACHALASIA

Failure of the lower esophageal sphincter to completely relax upon swallowing results in a functional obstruction and pressurization of the esophageal body. Defective esophageal emptying progressively leads to dilatation and tortuosity of the esophagus with loss of the peristaltic waveform. Overall deterioration of esophageal function and structure with time, and the fact that peristalsis can return after surgical myotomy, suggest that the motor abnormalities secondary to esophageal outflow obstruction may be reversible and that early definitive treatment of achalasia is essential to preserve esophageal function^[1].

Achalasia has an insidious onset. Dysphagia and food regurgitation are the two major presenting symptoms of the disease. Nocturnal regurgitation often leads to recurrent episodes of aspiration pneumonia. In about 40% of patients the diagnosis of achalasia is delayed by the reported symptoms of chest pain and heartburn simulating gastroesophageal reflux disease. As the disease progresses, inability to swallow causes malnutrition and weight loss. Squamous-cell carcinoma of the esophagus appears to develop with greater frequency in patients with long-standing achalasia than in the normal population^[2].

The most sensitive tests for detecting achalasia are esophageal manometry and barium swallow videofluoroscopy. Aperistalsis and incomplete lower esophageal sphincter relaxation are the typical manometric features. Radiological abnormalities include aperistalsis, esophageal dilatation, and minimal lower esophageal sphincter opening with a bird-beak appearance. Endoscopic assessment is important to exclude the diagnosis of malignancy-induced secondary achalasia, often referred to as pseudoachalasia, before invasive therapies are implemented. Clinical features suggesting a tumor of the gastroesophageal junction are a short duration of dysphagia, a significant weight loss, and an elderly patient. Since adenocarcinoma of the cardia may present endoscopically as an infiltrating lesion with apparently normal mucosa, CT scan, endoscopic ultrasonography, and even exploratory laparoscopy should be used liberally in this subgroup of patients^[3].

MANAGEMENT OF ACHALASIA

Endoscopic dilation versus surgery

Treatment of achalasia is palliative and is directed toward elimination of the outflow resistance caused by the abnormal lower esophageal sphincter function without creating excessive gastroesophageal reflux. Sustained symptomatic relief of dysphagia can be achieved by endoscopic pneumatic dilation or by surgical myotomy. Retrospective studies have shown better results with myotomy performed by an experienced surgeon, and in the only prospective randomised trial myotomy gave better long-term results compared with pneumatic dilatation^[4]. A recent study of 217 patients who received a median of 4 pneumatic dilations over a 12-year period reported a long-term therapeutic success of only 50%^[5]. Over the past 15 years, the advent of minimally invasive technology has made surgery a more attractive option as a first-line therapy to both patients and referring physicians^[6]. It has been shown that pneumatic dilation is 72% effective versus 92% for the laparoscopic Heller myotomy. In addition, laparoscopic techniques have sharply reduced surgical morbidity^[7] (Table 1).

Principles of surgical therapy

The basic goal of surgery in the treatment of achalasia is the extramucosal division of both layers of the esophageal muscularis propria and of the oblique gastric fibers, the so-called Heller myotomy. For many years this operation has been performed through a laparotomy, often with the addition of a fundoplication, or through a left thoracotomy. The extent of the myotomy in the stomach, which in part depends on the type of surgical access, has long been a matter of controversy. Ellis, the pioneer of the transthoracic approach, advocated a limited (< 1 cm) gastric myotomy without an antireflux procedure. However, at a very late follow-up the level of symptomatic improvement markedly deteriorated over time with this approach, and the rate of excellent results (i.e., asymptomatic patients) progressively decreased from 54% at 10 years to 32% at 20 years^[8]. The majority of European surgeons have instead favored the transabdominal approach which includes a longer gastric myotomy (1 to 2 cm) and a fundoplication to protect the esophagus from iatrogenic gastroesophageal reflux. Another debated issue has been the direction of the myotomy over the stomach, which may be closer to the lesser curve or to the greater curve, and, as a consequence, can divide the semicircular (clasp) fibers and the oblique (sling) fibers in distinct proportions^[9].

A third major issue is the opportunity to add an antireflux repair to the myotomy. The rationale for considering a concomitant fundoplication is the assumption that a well performed surgical myotomy renders the lower esophageal sphincter incompetent. Furthermore, gastric contents propelled retrograde into an aperistaltic esophagus are not effectively cleared, thus magnifying the damage caused by postoperative reflux. The majority of surgeons worldwide presently favor a transabdominal approach and add to the myotomy a partial fundoplication, either a 180 degree anterior (Dor) or a 270 degree posterior fundoplication

Table 1 Pooled response rate of achalasia treatments^[7]

Therapy	n	Weighted response rate (%)	Weighted follow-up (yr)
Botulinum toxin	149	32	1.1
Pneumatic dilation	1276	72	4.9
Heller myotomy			
Thoracotomy	1221	84	5
Laparotomy	732	85	7.6
Laparoscopy	171	92	1.2

(Toupet). The partial fundoplication, as opposed to the 360 degree Nissen repair, does not cause significant resistance to esophageal emptying, therefore reducing the risk of postoperative dysphagia. The main advantage of an anterior Dor fundoplication is that this technique does not require mobilization of the distal esophagus and cardia, and provides a natural patch over the denuded esophageal mucosa. Overall, there has been a greater than 90% symptomatic relief and a less than 10% incidence of pH proven gastroesophageal reflux with this approach^[10]. This operation should be proposed as the first therapeutic option also in patients with sigmoid-shaped esophagus, although the chances of symptomatic relief are reduced in such circumstances and in some of these individuals an esophagectomy can eventually be required.

Minimally invasive surgery for achalasia

During the early times of the minimally invasive surgical approach, both laparoscopy and thoracoscopy have been used to perform a Heller myotomy. However, it soon became clear that laparoscopy offers several inherent advantages, including superior visualization of the gastroesophageal junction, a single lumen endotracheal intubation, the ability to add an antireflux procedure, and a shorter hospital stay. In addition, laparoscopy showed a better symptomatic outcome and a lesser incidence of postoperative gastroesophageal reflux^[11].

The first laparoscopic Heller procedures were performed in England and in Italy during the early 1990s^[12,13]. Compared to the open abdominal approach, a technical innovation introduced in the laparoscopic era has been the use of intraoperative endoscopy as a means of precisely identifying the gastroesophageal junction, checking for the completeness of the myotomy and testing for the presence of occult perforations. During the early phase of the learning curve, a Rigidflex balloon dilator was also used to distend the cardia in order to facilitate division of all residual muscle fibers^[14]. Subsequently, transillumination and air inflation provided by the endoscope were considered appropriate to assist the myotomy^[15]. Further demonstration of the effectiveness of intraoperative endoscopy came from a study showing that endoscopic and laparoscopic criteria were discordant in the identification of the esophagogastric junction in 58% of the cases, the cardia being in all these cases at a more distal site with endoscopic criteria. As a consequence, based on the laparoscopic appearance, the surgeon may err by underestimating the caudal extent of the myotomy and

can perform a too short myotomy on the gastric side^[16]. Although we are still convinced of the effectiveness of intraoperative endoscopy during the learning curve of this operation and during reoperative surgery, we have now discontinued this practice in our high-volume referral center where between 15 and 20 patients per year undergo Heller myotomy as a primary treatment for achalasia.

It should also be noted that a common reason for an incomplete myotomy on the gastric side is the fear of producing a mucosal injury, which typically occurs just at the esophagogastric junction. The mean reported rate of mucosal perforation is about 5%, but the frequency is largely dependent on surgeon's experience. There are no consequences if the mucosal injury is detected intraoperatively and repaired with interrupted stitches^[15].

The principle of limited surgical dissection of the cardia, already advocated by some surgeons in the open era^[10], has been successfully reproduced in many centers with the minimally invasive laparoscopic approach. Dissection is limited to the anterior and lateral attachments of the phrenoesophageal membrane as this allows enough space to perform the myotomy and helps to prevent the occurrence of postoperative gastroesophageal reflux. Only in patients with an associated hiatal hernia or epiphrenic diverticulum the distal esophagus is encircled and a posterior crural repair is performed. By grasping the cardia and pulling it in a caudal direction, the myotomy is started on the distal esophagus using a L-shaped electrocautery hook until the submucosal plane is identified. The myotomy is carried out for about 6 cm on the esophagus, toward the left of the anterior vagus nerve, and up to 2 cm on the gastric side to include the oblique fibers.

Despite the minimal surgical dissection herein described, an antireflux repair added to the Heller myotomy appears to be beneficial and is nowadays supported by the majority of esophageal surgeons. The anterior Dor fundoplication is a technically simple procedure, quick and safe to perform laparoscopically. It does not require mobilization of the cardia, and places a gastric fundic patch over the myotomy site to protect the mucosa and prevent re-healing of the myotomy. Reconstruction of the angle of His is first performed by suturing the medial wall of the fundus to the left edge of the myotomy and to the left crus of the diaphragm. The Dor fundoplication is fashioned by folding the anterior fundic wall over the myotomized surface and securing the stomach to the right crus of the diaphragm and to the right edge of the myotomy with interrupted stitches. It is important to pay attention to the geometry of the fundoplication in order to avoid undue tension and to provide a uniform patch over the myotomized esophagus, but usually there is no need to divide the short gastric vessels. The Heller-Dor operation is carried out in about one hour by an expert surgeon. A gastrographin swallow study is performed the following day to check esophageal transit and to rule out leaks. A soft diet is then permitted, and the patient is usually discharged home on postoperative d 2 or 3.

Studies including large number of patients have shown that an extramucosal myotomy of the esophagus and cardia combined with an anterior fundoplication can be

Table 2 Twenty-four h pH data of patients submitted to Heller myotomy and Heller myotomy plus Dor fundoplication in a randomized study^[24]

Parameter	Heller (n = 18)	Heller-Dor (n = 21)	P value
Time (%) pH < 4 upright	8.1 + 10.4	0.8 + 1.1	0.015
Time (%) pH < 4 supine	9.1 + 18.3	2.0 + 6.9	0.002
No. episodes pH < 4	113 + 128	25 + 6.9	0.001
No. episodes pH < 4 for ≤ 5 min	3.4 + 4.7	0.5 + 1.6	0.001

performed safely and effectively through laparoscopy, with clinical and functional results similar to that obtained with the open transabdominal approach and relief of dysphagia in more than 90% of patients^[17-22]. Although previous endoscopic treatments, such as balloon dilatation or intrasphincteric botulinum toxin injection, may cause submucosal scarring at the esophagogastric junction resulting in a more difficult surgical procedure and an increased operative morbidity, no statistically significant differences as far as concern the clinical outcome have been reported^[15].

Radiologic and manometric studies after the laparoscopic Heller-Dor operation have shown a significant decrease of the internal diameter of the thoracic esophagus and of the resting lower esophageal sphincter pressures^[19]. In a recent report, at a minimum follow-up of 6 years after the operation, about 82% of patients were satisfied with the treatment and were able to eat normally; more than a half of the symptomatic recurrences occurred during the first year of follow-up and were effectively treated with pneumatic dilations. Nine (12.7%) patients either had abnormal acid exposure on postoperative 24-h pH study or were on treatment with proton-pump inhibitors for reflux symptoms; however, in none of these individuals did endoscopy reveal more than grade A esophagitis^[23].

An important preoperative factor affecting the outcome of the laparoscopic operation is the magnitude of the resting pressure of the lower esophageal sphincter. On a multivariate analysis, only a high resting pressure prior to surgery was a predictor of resolution of dysphagia. Interestingly, all patients with a preoperative sphincter pressure greater than 36 mmHg had their dysphagia resolved after surgery^[24].

The issue of whether an antireflux procedure should be added to the Heller myotomy has long been controversial and supported only by personal feelings and retrospective studies. Recently, a randomised double-blind clinical trial comparing the outcome of myotomy plus Dor fundoplication versus myotomy alone has shown that the former operation is superior in terms of reflux control (Table 2). The addition of a Dor fundoplication reduces the risk of pathologic gastroesophageal reflux by 9-fold as tested by 24-hour esophageal pH monitoring. Even the median esophageal acid exposure was lower in the Heller-Dor group, indicating that the few unfortunate individuals in whom reflux might occur are easily managed with

medical therapy^[25].

The predominant mechanism of failure after the Heller operation is an incomplete distal myotomy. In such circumstances, pneumatic dilatation can represent a reasonable therapeutic option when dysphagia is mild. In patients with major symptoms, revisional laparoscopic surgery with repeat myotomy and fundoplication is feasible and effective with a low morbidity rate^[26]. Intraoperative endoscopy is helpful as a guide during dissection to identify the cleavage plans and to clearly recognize the gastroesophageal junction. When a properly performed myotomy has failed in a patient with sigmoid esophagus, and the redundant supradiaphragmatic esophageal loop still interferes with emptying, a transthoracic or a transhiatal esophagectomy is the treatment of choice^[27].

It has recently been suggested that a laparoscopic myotomy extended for 3 cm on the gastric side can result in better dysphagia scores and in fewer additional procedures to treat recurrent dysphagia, provided that a Toupet fundoplication is added to the procedure^[28]. However, this improved outcome should be balanced against the risk of severe gastroesophageal reflux disease induced by the longer gastric myotomy and the necessity to fully mobilize the cardia to perform a posterior fundoplication. Interestingly, a very long-term study of 67 patients who underwent an open Heller-Dor operation and were followed for more than 20 years has shown a progressive clinical deterioration of results and a 22.4% failure rate due to an increased esophageal acid exposure and development of Barrett's esophagus^[29], suggesting once again that surgical therapy for achalasia involves a very delicate balance between relief of outflow obstruction and destruction of the antireflux barrier.

Robot-assisted Heller operation has been performed with satisfactory outcomes and no mucosal perforation^[30]. Operative times were similar to those of standard laparoscopic operation after the first 30 cases in a multicenter study^[31]. No data from randomised clinical trials have been reported yet. At present time, despite the advantage of the three-dimensional view, elimination of tremor, and 360 degree of freedom of movement of the robotic arms, the role of this technology in the management of esophageal achalasia remains to be determined.

CONCLUSIONS

The management of achalasia has changed significantly over the past 15 years. Minimally invasive surgery has influenced the management of esophageal disease more than any other gastrointestinal disorder and in a manner similar as in the therapy of cholelithiasis. In several institutions around the world, the laparoscopic surgical myotomy has replaced pneumatic dilation as the initial therapy of choice for achalasia. Current evidence from the literature suggests that it is conceivable to extend the Heller myotomy on the gastric side for about 2 cm to encompass the oblique fibers without fear of inducing significant gastroesophageal reflux, provided that a minimal antero-lateral dissection of the phrenoesophageal membrane is performed and a Dor fundoplication is routinely added to the myotomy.

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EDITORIAL

New methods for the management of gastric varices

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Abstract

Bleeding from gastric varices has been successfully treated by endoscopic modalities. Once the bleeding from the gastric varices is stabilized, endoscopic treatment and/or interventional radiology should be performed to eradicate varices completely. Partial splenic artery embolization is a supplemental treatment to prolong the obliteration of the veins feeding and/or draining the varices. The overall incidence of bleeding from gastric varices is lower than that from esophageal varices. No studies to date have definitively characterized the causal factors behind bleeding from gastric varices. The initial episodes of bleeding from esophageal varices or gastric varices without prior treatment may be at least partly triggered by a violation of the mucosal barrier overlying varices. This is especially likely in the case of varices of the fundus. In view of the high rate of hemostasis achieved among bleeding gastric varices, treatment should be administered in selective cases. Among untreated cases, steps to prevent gastric mucosal injury confer very important protection against gastric variceal bleeding.

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Key words: Gastric varices; Esophageal varices; Bleeding

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INTRODUCTION

Bleeding from esophageal varices (EVs) or gastric varices (GVs) is a catastrophic complication of chronic liver disease. Bleeding from GV is generally thought to be more severe than bleeding from EVs^[1], but it occurs

less frequently^[2-4]. Though many recent developments have improved the outcome of treatments for GV, no consensus has been reached on the optimum treatment. In this paper we review the pathomorphology, hemodynamics, risk factors for bleeding, and treatments for GV. In the esophagogastric varices grading system of the Japan Society for Portal Hypertension^[5], the varices are evaluated based on color (white [Cw], and blue [Cb]), form (small and straight [F1], nodular [F2], and large or coiled [F3]), and the red color sign (RC0-3). GV are divided into cardiac varices (Lg-c), fundal varices (Lg-f), and varices involved both the cardia and fundus (Lg-cf). In this review, GV are divided into two categories and described accordingly: Lg-c (cardiac varices: CVs) and Lg-cf or Lg-f (fundal varices: FVs).

PATHOMORPHOLOGY OF GV

Arakawa *et al*^[6] reported that CVs are supplied by the left gastric vein (cardiac branch), a vessel which enters the stomach wall in the cardia at a point 2 to 3 cm from the esophagogastric junction and diverges into a profusion of branches running throughout the cardia. Some of these veins become markedly dilated, acquiring the features of varices. Most veins in the cardia diverge into parallel veins from the esophagogastric junction as the flow becomes hepatofugal. However, Others will dilate, wind through the submucosa, and directly join EVs. Histologically, nearly the entire cross-section of the wall is the varix itself. The varices are covered by thinning layers of serosa and mucosa through which they can ultimately be seen.

The angio-architecture of a FV is quite different from that of a CV. Most FVs are supplied by the short gastric vein, though in some cases the blood is fed from the posterior or left gastric vein. Thus, the vascular anatomy of a FV is something like a splenorenal shunt running through the stomach wall. Bleeding from an EV most commonly occurs in the "critical area" 3 cm proximal to the esophagocardiac junction. Fine longitudinal veins in the lamina propria originate at the esophagocardiac junction and run in the lamina propria toward this critical area. EVs consist of multiple dilated veins. Those that rupture are usually located in the lamina propria^[7].

In the stomach, unlike its counterpart the esophagus a large winding vein runs through the submucosa without causing varicose veins to pile up. The ruptures in CVs occur in the submucosa, where they disrupt the muscularis mucosae and lamina propria mucosae. The mucosal layer covering a FV is somewhat thicker than that covering an EV. The difference between a CV and a FV lies in the

caliber of the varicose vein and the degree of vascular anastomosis. Most FVs are supplied *via* the short gastric vein, though some are fed by the posterior or left gastric vein. Anastomosis of FVs is generally uncommon. The varices within the wall penetrate the muscle layer and wind through the submucosal layer, where they displace and attenuate the muscularis mucosae and propria mucosae. The varicose veins protrude into the gastric lumen.

The lamina muscularis mucosa in the esophageal mucosa is loose, and the venous pressure in the submucosa is transmitted through communicating branches to the veins in the lamina propria. The lamina muscularis mucosa in the gastric mucosa, on the other hand, is tightly integrated with the lamina propria^[8].

The red color sign is an elevated red area which has proven to be important in portending variceal bleeding. The histological manifestation is a thinning of the epithelial layer. The North Italian Endoscopic Club for the Study and Treatment of Esophageal varices^[9] published a report establishing that the red color sign on EVs is predictive of bleeding. It remains unclear whether the endoscopic red color sign in the stomach has the same significance as the red color sign in the esophagus. In the latter case does it denote a thinning of the epithelial layer. The varix in the submucosa of the stomach is covered by the muscularis mucosae and propria mucosae. This generally confers an appearance different from that typical of the thinning epithelial layer of the esophagus^[6].

HEMODYNAMICS OF GVs

The portal hemodynamics of GVs should be evaluated in all patients with these varices to determine the most appropriate treatment. CVs are supplied by the left gastric vein (cardiac branch), a vessel which enters the stomach wall in the cardia at a point 2 to 3 cm from the esophagogastric junction and diverges into a profusion of branches running throughout the cardia. The main veins feeding the FVs are the left gastric vein (51%), posterior gastric vein (30%), and short gastric vein (69%). The principal drainage veins for the FVs are the gastro-renal shunt (87%) and gastric-inferior phrenic vein shunt (16%), though about 1% of FVs reported to communicate with neither^[10]. FVs are more frequently supplied by the short and posterior gastric veins than CVs. Concomitant collaterals such as EVs, para-esophageal veins, and paramesenteric veins are additionally observed in nearly 60% of FVs.

RISK FACTOR FOR BLEEDING FROM GVs

The incidence of variceal bleeding in patients who have never received treatment for EVs has been reported to range from 16 to 75.6%^[11,12]. The incidence of bleeding from GVs stands at 25%^[2], while cumulative bleeding rates from FVs at 1, 3, and 5 years have been estimated at 16%, 36%, and 44%, respectively^[13]. Thus, the overall incidence of bleeding from GVs is lower than that from EVs^[2]. In an earlier study on the natural course of GVs in 52 patients, our group treated bleeding from GVs in 4 patients over a mean follow-up period of 41 mo. Hemorrhage

was successfully halted in all 4 of these patients. The cumulative bleeding rates at 1, 3, and 5 years were 3.8%, 9.4%, and 9.4%, respectively. Three of the 4 patients were free of erosive gastritis and gastric ulcer at the time of entry into the study, though ulcers or erosions were found at the bleeding points of the GVs in all 4 when the varices ruptured. There were no significant differences in patient characteristics with ruptured *versus* non-ruptured GVs when the patients entered the study^[4].

The endoscopic risk factors for bleeding from EVs include the presence of raised red markings, cherry-red spots, blue color, and large size^[14]. The risk factors for bleeding from GVs have yet to be characterized. In another study, our group examined 70 cirrhotic patients with first-time bleeding from EVs or GVs without prior treatment^[15]. The red color sign was more common in EVs than in CVs or FVs ($P < 0.0001$). Mucosal erosion over the varices at the site of bleeding was more common in CVs ($P < 0.0005$) and FVs ($P < 0.0001$) than in EVs. An ulcer at the bleeding point was more common in CVs ($P < 0.01$) and FVs ($P < 0.0001$) than in EVs. Gastric ulcer was more common in CVs ($P < 0.05$) and FVs ($P < 0.001$) than in EVs. Erosive gastritis was more common in FVs ($P < 0.02$) than in EVs. The red color sign, a strong risk factor for the ruptures frequently encountered in EVs, was completely absent in the FVs. All of the CVs manifesting the red color sign communicated with EVs which manifested the red color sign themselves. This might have been due to the pronounced thickness of the mucosal layer overlying the FVs. FVs are usually two or three times larger than EVs and drain directly into an extremely dilated left gastric or posterior gastric vein^[16]. The volume of blood flow through a FV therefore usually exceeds that through an EV. Gastric ulcers that develop over GVs represent violation of the protective layer of gastric mucosa. Violation of the mucosal barrier overlying GVs place patients at risk of massive bleeding, especially when FVs are involved. Violations of this type could be an important precondition leading to variceal hemorrhage.

TREATMENT OF GVs

Treatment modalities for GVs include balloon tamponade, endoscopic treatment, embolization, and surgery.

Balloon tamponade

Balloon tamponade with the Sengstaken-Blakemore or the Linton-Nachlas tube is an emergent procedure for active hemorrhaging from GVs. The procedure is effective in the short term, but permanent hemostasis is obtained in fewer than 50% of cases^[17,18].

Endoscopic treatment

Two endoscopic treatment modalities are used for the treatment of esophagogastric varices: endoscopic injection sclerotherapy (EIS) and endoscopic variceal ligation (EVL)^[19-26]. EIS can be accomplished by either intravariceal EIS or extravariceal EIS^[21-23,26]. In the treatment of EVs, intravariceal EIS obliterates both the interconnecting perforating veins and the veins feeding the EVs. Most veins in the cardia become parallel veins from the

esophagogastric junction at the point at which the flow becomes hepatofugal. Nearby, however, a number of dilated winding cardiac veins run through the submucosa and directly join the EVs. This makes it possible to treat most CVs concomitantly with EVs when correcting the latter by intravariceal EIS.

EIS and EVL are both effective for the treatment of bleeding from EVs and CVs. EIS has been less successful in the treatment of bleeding from FVs, however. When used with 1% polidocanol, 5% ethanolamine oleate iopamidol (EOI), or thrombin for this purpose, EIS has a high rate of operative mortality^[27-29]. Fortunately, the rate of initial hemostasis has been significantly improved since the introduction of *N*-butyl-2-cyanoacrylate (Histoacryl) as the sclerosant in EIS^[30,31]. We should note, however, that bleeding from the GV's injection site and rebleeding from the rupture point have been reported in patients receiving EIS^[2,29].

While EVL is generally safe and effective for the treatment of CVs and FVs^[32], it sometimes causes deep or extensive ulcers and increases the risk of ensuing ulcer hemorrhage or secondary bleeding^[33]. FVs are usually twice or three times larger than EVs and are directly connected to an extremely dilated left gastric or posterior gastric vein^[16]. The volume of blood flow through an FV therefore usually exceeds that through an EV^[34]. A mucosal injury remains on the varices after endoscopic treatment. If the blood flow in the varices cannot be stopped completely, bleeding may recur at the site of this mucosal injury. This underlines the importance of ensuring the complete obliteration of the blood flow when treating FVs endoscopically. It may be dangerous to treat FVs by EVL alone.

GVs have also been treated by a combined endoscopic method using a detachable snare and simultaneous EIS and O-ring ligation^[35]. This technique is not yet in widespread use, however. Our group published a report on the treatment of ruptured GV's by EIS with Histoacryl followed by O-ring ligation (endoscopic scleroligation: EISL)^[24]. EISL was developed as a treatment modality for EVs to prevent bleeding from the injection site during needle removal^[21,36]. When treating GV's by EIS with Histoacryl, the immediate freezing of the Histoacryl around the needle hinders the removal of the needle after the injection. In some cases, bleeding from the GV injection site or rebleeding from the rupture point also occurs^[2,29]. Our group used the EISL procedure to treat ruptured GV's with punctures near the rupture points by simultaneous ligations of the injection sites and rupture points. EISL effectively stopped the bleeding from the GV's, enabled swift and easy needle removal, and successfully eliminated both bleeding from the injection site and rebleeding from the rupture point. An O-ring was placed at the point of the EISL injection with Histoacryl and left in position for a long time. As of this writing, EISL with Histoacryl is considered the most promising treatment for hemorrhaging GV's.

Interventional radiology (IVR)

The portal hemodynamics of GV's, the main feeding veins from the portal system, and the main drainage veins into

the vena cava should be determined in all patients with the GV. Angiography can determine the hemodynamics of the GV simultaneously during treatment by embolization.

Transportal obliteration: Two methods have been used to obliterate the feeding veins of GV's: percutaneous transhepatic obliteration and trans-ileocolic vein obliteration. The catheter is inserted directly into the portal vein, the portal circulation is visualized by portography, a balloon catheter is inserted selectively into the inflow site of the feeding veins for the varices, the balloon is inflated, and a test dose of a contrast medium is injected to determine the optimal volume of sclerosant fluid. Five percent EOI and/or 500 g/L glucose is injected to obliterate the feeding vein, then steel coils are used to complete the obliteration^[37]. The procedure is quite effective, though only transportal obliteration is sometimes incomplete, especially in FVs.

Balloon-occluded retrograde transvenous obliteration (BRTO): BRTO is a notable IVR procedure developed specially for the treatment of FVs. The technique is performed by inserting a balloon catheter into the outflow shunt (gastric-renal shunt or gastric-inferior phrenic vein shunt) *via* the femoral or internal jugular vein. Any existing collateral veins are treated with coils, absolute ethanol, or a small amount of 5% EOI. The balloon is inflated and a test dose of contrast medium is injected to determine the optimal volume of the sclerosant solution. Five percent EOI is slowly injected through the catheter until the shunt is filled with the sclerosant fluid. The catheter is removed after 24 h of balloon occlusion^[38-40]. A remarkably high rate of FV eradication or reduction in FV size can be expected if the BRTO procedure is technically successful. Indeed, long-term eradication of treated FVs without recurrence is achieved in most patients^[38,41]. Kanagawa *et al*^[38] confirmed eradication of FVs in 97% of 32 patients treated by this procedure, and no FVs recurred in any of those patients within an average follow-up period of 14 mo. In earlier reports, the eradication rate of FVs exceeded 89% and the recurrence rate was less than 7%. In light of the minimal invasiveness and high safety of the procedure, BRTO is applicable not only for elective cases, but also for emergency cases with FVs.

FV treatment by BRTO has two significant effects, namely, eradication of the FVs themselves and obliteration of the unified portal-systemic shunt. Thus, most of the benefits and adverse effects of BRTO are related to obliteration of the unified portal-systemic shunt. Benefits such as decreased blood ammonia levels and improved porto-systemic encephalopathy are sometimes observed. Possible adverse effects include transient ascites, increases of ascites, pleural effusion, and the appearance of EVs manifesting the red color sign. These adverse effects may be due to elevation of the portal pressure in reaction to the occlusion of the portal-systemic shunt.

Partial splenic artery embolization (PSE): The femoral artery approach is used for super-selective catheterization of the splenic artery. The catheter tip is placed as distally as possible in either the hilus of the spleen or in an intrasplenic artery. Embolization is achieved by injecting 2-mm cubes of gelatin sponge suspended in a saline solution containing antibiotics^[42,43]. PSE has been

performed to treat hypersplenism, EVs, GV, portal hypertensive gastropathy, pancreatic carcinoma, and porto-systemic encephalopathy^[37,43-53]. Our group evaluated PSE in a long-term study of 26 patients with hepatic cirrhosis alongside 26 patients who did not undergo the PSE procedure^[42]. The red blood cell counts of the PSE (+) group increased significantly by 6 mo after the procedure and remained increased for up to 7.5 years. The platelet counts peaked only 2 wk after PSE and gradually fell thereafter. Even so, the platelet counts remained significantly higher than the pre-PSE level for up to 8 years. No significant changes were observed in the aspartate aminotransferase and alanine aminotransferase activities in serum during the follow-up. Cholinesterase activity was increased significantly by 6 mo after PSE and remained increased for more than 7 years. The serum albumin concentration increased significantly from 6 mo after PSE and the level remained significantly increased for 6 years. Survival did not differ between the PSE (+) and PSE (-) groups. PSE, a non-surgical treatment, can benefit patients with cirrhosis by improving the capacity of hepatic protein synthesis and conferring protection against hemorrhage due to thrombocytopenia.

Combination modalities with IVR: Our group also reported the long-term results of PSE as supplemental treatment for portal-systemic encephalopathy. We divided 25 patients with portal-systemic encephalopathy due to portal-systemic shunts into two groups, one treated by transportal obliteration and/or BRTO of portal-systemic shunt, followed by PSE (PSE (+) group; $n = 14$), the other treated by transportal obliteration and/or BRTO of the portal-systemic shunt without PSE (PSE (-) group; $n = 11$). The serum ammonia levels and grades of encephalopathy were lower in the PSE (+) group than in the PSE (-) group at 6, 9, 12, and 24 mo after treatment. Obliteration of the portal-systemic shunt raised the portal venous pressure in every case. As all of the patients had cirrhosis, the portal-systemic shunt drainage reduced portal hypertension and the obliteration of the portal-systemic shunt led to portal congestion and increased portal venous pressure. Our study thus confirmed the benefits of obliteration of the portal-systemic shunt by PSE in patients with portal-systemic encephalopathy^[43].

PSE is performed incrementally during the monitoring of the portal pressure in order to reduce the portal venous pressure to the level measured before obliteration of the veins feeding and/or draining the GV^s^[22,42,43,49,54]. PSE is a supplemental modality to prolong the effect of obliteration of the veins feeding and/or draining the GV^s.

Combination of endoscopic treatment and IVR

Treatment of GV^s solely by endoscopic modalities or by IVR is occasionally incomplete. Our group previously reported that combined treatments with IVRs and endoscopic modalities had significant impacts on long-term rebleeding and retreatment rates in patients with EV^s or GV^s^[37,48,50,51]. In elective cases, complete GV treatment should be administered in order to prevent rebleeding with greater assurance.

Surgery

A number of surgical procedures have been developed to manage esophagogastric varices. These can be classified as shunting and nonshunting procedures. The goal of shunting is to reduce the incidence of variceal bleeding by lowering the pressure in the portal system using a portal-systemic shunt. While the standard portocaval shunt effectively reduces the incidence of variceal bleeding, impairment of the hepatic protein metabolism in patients undergoing the procedure frequently leads to the development of hepatic encephalopathy due to hyperammonemia^[55-57]. The distal splenorenal shunt (DSRS) was developed by Warren *et al.*^[58] in 1967 as a way to preserve portal blood flow through the liver while lowering variceal pressure. The hope, in developing this approach, was to prevent both bleeding and hyperammonemia. While DSRS effectively prevents rebleeding, patients who undergo DSRS still can develop hyperammonemia. Our group responded by designing a DSRS with a splenopancreatic disconnection and gastric transection, modifications to prevent the loss of shunt selectivity. This modified DSRS has been proved to reduce the incidence of postoperative hyperammonemia^[59].

As an alternative to shunting, Hassab^[60] and Sugiura *et al.*^[61] developed a method of gastro-esophageal decongestion and splenectomy for the treatment of varices. The Hassab operation devascularizes the distal esophagus and proximal stomach. Splenectomy, selective vagotomy, and pyloroplasty can be performed concomitantly with the procedure. Sugiura *et al.*^[61] developed a method of esophageal transection for patients with GV^s and EV^s. Sugiura's method is performed concomitantly with the Hassab operation to divide and reanastomose the distal esophagus in order to disrupt the blood supply to the EV^s. While both procedures may solve the problem of hepatic encephalopathy, varices are likely to recur earlier than they are in patients undergoing DSRS^[62].

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REVIEW

Complementary analysis of microsatellite tumor profile and mismatch repair defects in colorectal carcinomas

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counseling if appropriate. MSI is an excellent functional and prognostically useful marker, whereas MMR immunohistochemistry can guide gene sequencing.

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Key words: Colon carcinoma; Microsatellites; Mismatch Repair; Hereditary non-polyposis colon cancer

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Abstract

Microsatellite instability (MSI) is a prognostic factor and a marker of deficient mismatch repair (MMR) in colorectal adenocarcinomas (CRC). However, a proper application of this marker requires understanding the following: (1) The MSI concept: The PCR approach must amplify the correct locus and accurately identify the microsatellite pattern in the patient's normal tissue. MSI is demonstrated when the length of DNA sequences in a tumor differs from that of nontumor tissue. Any anomalous expansion or reduction of tandem repeats results in extra-bands normally located in the expected size range (100 bp, above or below the expected product), differ from the germline pattern by some multiple of the repeating unit, and must show appropriate stutter. (2) MSI mechanisms: MMR gene inactivation (by either mutation or protein down-regulation as frequently present in deep CRC compartments) leads to mutation accumulation in a cell with every cellular division, resulting in malignant transformation. These mechanisms can express tumor progression and result in a decreased prevalence of aneuploid cells and loss of the physiologic cell kinetic correlations in the deep CRC compartments. MSI molecular mechanisms are not necessarily independent from chromosomal instability and may coexist in a given CRC. (3) Because of intratumoural heterogeneity, at least two samples from each CRC should be screened, preferably from the superficial (tumor cells above the muscularis propria) and deep (tumor cells infiltrating the muscularis propria) CRC compartments to cover the topographic tumor heterogeneity. (4) Pathologists play a critical role in identifying microsatellite-unstable CRC, such as occur in young patients with synchronous or metachronous tumors or with tumors showing classic histologic features. In these cases, MSI testing and/or MMR immunohistochemistry are advisable, along with gene sequencing and genetic

INTRODUCTION

Colorectal carcinoma (CRC) is generally classified into three categories, based on increasing hereditary influence and cancer risk^[1,2]: sporadic CRC (approximately 60% of cases and comprises patients with no notable family history and, by definition, with no identifiable inherited gene mutation that accelerates cancer development), familial CRC (approximately 30% of cases and refers to patients who have at least one blood relative with CRC or an adenoma, but with no specific germline mutation or clear pattern of inheritance), and hereditary CRC syndromes (approximately 10% of cases, which result from inheritance of a single gene mutation in highly penetrant cancer susceptibility genes). Although the last group has the lowest frequency, it has elucidated molecular mechanisms of carcinogenesis applicable to sporadic CRC^[3].

The microsatellite profile of sporadic CRC is a prognostically useful marker^[4-7]. Microsatellites are repeating DNA sequences of unknown function that are found throughout the genome^[8]. Microsatellite instability (MSI) is demonstrated when the length of DNA sequences in tumor and nontumor tissues is different and MSI has been identified in a wide variety of human tumors, due to defects in one of the DNA mismatch repair (MMR) genes, especially *MLH1* or *MSH2*^[1,9]. However, MSI presence alone does not establish a diagnosis of hereditary non-polyposis colon cancer (HNPCC) because MSI has also been identified in 10%-30% of sporadic CRC. Certain histological features also correlate with the presence of

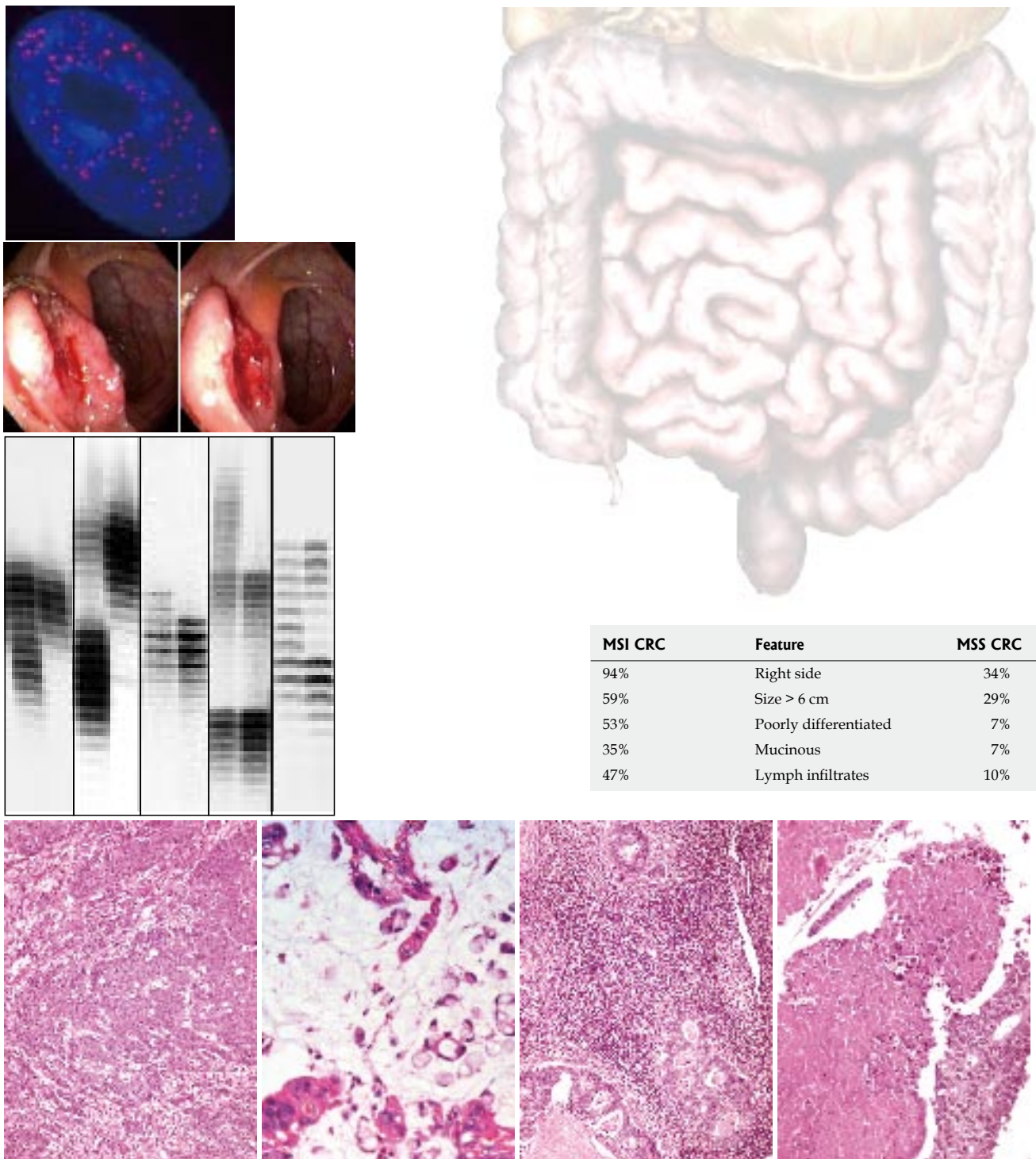


Figure 1 Microsatellite unstable CRCs are normally located proximal to the splenic flexure and reveal non-polypoid pattern. MSI can be demonstrated by fluorescence in-situ hybridization (FISH) or molecular techniques. Histopathologically, these tumors show solid growth, mucinous differentiation, prominent lymphocytic infiltrate, and no dirty necrosis^[10].

MSI in sporadic CRC (Figure 1)^[5,10-13], which can be key elements in the design of more effective therapeutic protocols^[12,13].

Both basic and clinical implications of MSI and MMR defects need to be considered in an appropriate context, which requires clarifying the definition of MSI, the biological consequences of tumor MSI, interference of intratumor heterogeneity on MSI detection, differences in clinical testing for MSI and for MMR defects, and MSI prognostic and therapeutic implications.

MSI DEFINITION AND CLINICAL TESTING FOR MSI

Any useful application of prognostic factors requires a reliable definition of the factor. Microsatellites belong to the family of highly polymorphic and repetitive non-coding DNA sequences that, although widely distributed in the human genome, are not uniformly spaced (underrepresented in subtelomeric chromosome regions). Microsatellites are useful molecular markers due to their

ubiquity, PCR typability (except for (dA)_n multimers, whose size polymorphisms are difficult to type), Mendelian co-dominant inheritance, and extreme polymorphism^[8], but their origin and function are not clear^[14]. They have been demonstrated to be very useful in cell lineage delineation, positional cloning, and several applications in forensic medicine^[15,16]. Microsatellite instability (MSI) is demonstrated when the lengths of DNA sequences in a tumor differ from those of nontumor tissue. MSI has been identified in a wide variety of human tumors.

Currently, tumor MSI analyses require molecular tests and the application of strict criteria. MSI can be defined as a change in any DNA sequence length due to either insertion or deletion of repeating units in a microsatellite within a tumor when compared to normal tissue^[17,18]. The tests must be run with appropriate controls (known positive and negative controls along with the patient's normal tissue)^[8,19], which are extremely important due to the non-exceptional presence of extra-bands. The PCR approach must amplify the correct locus and accurately identify the microsatellite pattern in the patient's normal tissue. Any anomalous expansion or reduction of tandem repeats due to MSI results in extra-bands. True extra-bands expressing tumor MSI are normally located in the expected size range (usually about 100 bp), are above or below the expected PCR product, differ from the germline pattern by some multiple of the repeating unit (e.g. delta 6 bases for dinucleotides), must show appropriate stutter (e.g. -2, -4 for dinucleotides), and are not present in the normal control. These tests should be carefully analyzed considering the following: (1) Sample homogeneity/heterogeneity can vary. Very small samples (even single cells) have been used in genetic analyses to avoid normal cell contamination. However, the lower the number of cells the higher the probability of technically-related abnormal results^[20], which can be partially resolved with appropriate methods. The high incidence of PCR artifacts using microdissected samples is related to the small concentration of target DNA, fixation induced changes of DNA, and conditions in the amplification of repetitive sequences (especially for those CG-rich sequences) favouring misannealing and hairpin formation. Appropriate modifications to avoid the above conditions will significantly improve the reproducibility of LOH and MSI tests in microdissected samples^[21]. (2) Appropriate controls are necessary for every step of the molecular tests to avoid false results. Sufficient levels of amplification with all markers should be obtained to detect low amounts of shifted microsatellites. (3) PCR bias against one allele (especially the larger one in a pair) can result in preferential amplification of the other allele (usually the smaller in a pair), which is the so-called artifactual allele dropout^[22,23]. An appropriate extraction method, providing DNA of quality^[24], and PCR designs including both long denaturation and extension in the first three cycles and 7-deazadGTP in the amplification mixture to improve the amplification of CG-rich DNA regions, will be reasonably helpful in avoiding that bias^[8,19,21,23,25]. (4) The number of polymorphic DNA regions agreed to at the NCI consensus conference includes a primary panel of at least 2 mononucleotide

Table 1 False negative in antigen positive neoplasms, comparative features of microsatellite unstable sporadic adenocarcinoma and hnpcc colon carcinomas

	MSI-H sporadic adenocarcinomas	HNPCC adenocarcinomas
Patient age	Older	Younger
Number of tumors	Single	Single/Multiple (synchronous/metachronic)
Colonic distribution	Right colon	Right colon
Histological clues	Poorly differentiated, medullary type Crohn-like inflammation	Poorly differentiated, medullary type Crohn-like inflammation
Mechanism of MMR deficiency	MLH1 promoter hypermethylation	Inactivating germline mutation of MMR proteins
Tumor prognosis	Better than MSI-L/MSS sporadic adenoca	Better than MSI-L/MSS sporadic adenoca

and 3 dinucleotide microsatellites, along with 19 alternate loci (both mono- and dinucleotides)^[26]. The choice of microsatellite markers is important in MSI testing, with the examination of mononucleotide repeats being sufficient for detection of MMR deficient tumors, whereas instability only in dinucleotides is characteristic of MSI-L/MMR-positive tumors^[27]. Depending on the number of abnormal loci from the total analyzed, the cases are classified into MSI-high ($\geq 30\%$ -40% of abnormal loci), MSI-low ($< 30\%$ -40% of abnormal loci), and MSS (no abnormal loci).

Which patients should be tested? The neoplasm histological features closely correlate with MSI and should be the key elements used to select sporadic CRC for MSI investigation^[5,10-13]. The sets of criteria for the clinical diagnosis of HNPCC appear under Clinical Testing for MMR defects. The implications of these analyses in sporadic and HNPCC carcinomas are compared in Table 1.

BIOLOGICAL CONSEQUENCES OF TUMOR MSI

Microsatellite-unstable CRC are biologically different and have a better survival rate than sporadic CRC when matched for cancer stage^[28-30]. The development of proximal and distal CRC involves partly different mechanisms associated with the MSI and the chromosomal instability (CIN) pathways^[31].

These two pathways are not always independent and some CRCs show a significant degree of overlap between these two mechanisms^[32]. In one study, 35% of CRC were microsatellite-unstable (21% MSI-low and 14% were MSI-high) and 51% of CRC had at least one LOH event, with the most frequent chromosomal losses observed on 18q (72.5%)^[32]. A significant degree of overlap between MSI and CIN pathways has been reported in that series: 6.5% of CRC with LOH were also MSI-high, and 23.3% of MSI-high CRC also had one or more LOH events. These data suggest that molecular mechanisms of genomic instability are not necessarily independent and may not

be fully defined by either the MSI or CIN pathways. In addition, a subgroup of CRCs showed no evidence of alterations in either of these two pathways of genomic instability (37.8% of microsatellite-stable CRCs had no LOH events identified)^[32], a situation similar to that reported in muscle-invasive transitional cell carcinomas of the bladder^[25].

MMR proteins normally identify and correct mismatched DNA sequences that can occur during DNA replication. An inactivating mutation in any of these genes leads to mutation accumulation in a cell with every cellular division, resulting in malignant transformation^[6,8,33-36]. Tumor progression in the deep compartments may be the result of MMR protein down-regulation, which would contribute to the following: (1) There is a decreased prevalence of aneuploid cell lines and K-RAS and B-RAF mutations detected in microsatellite-unstable CRC and in the deep compartments of sporadic CRC^[7,29,37,38]. Microsatellite-unstable CRCs tend to be diploid^[37,39], and to have lower DNA indices^[39]. Supporting these findings, the MMR protein down-regulation observed in the deep compartments of sporadic CRC has shown correlation with increased frequency of diploid DNA content^[40,41]. (2) Differential cell kinetics (proliferation and apoptosis) has been identified in superficial and deep compartments (above muscularis propria vs. muscularis propria) of sporadic CRC, which has demonstrated a close correlation with MMR protein expression (Figure 2)^[41,42]. Physiologic correlations between MMR protein expression and kinetic variables (mitotic figures, Ki-67 expression, ISEL index) were preserved in the superficial compartment only. In addition, G₂ + M phase fraction correlated with hMLH1 expression only in superficial compartments and hMSH2 expression only in deep compartments. Both the high cellular turnover and the maintained cell kinetic balance suggest that superficial compartments of sporadic CRC are expansile. In the deep compartments, the expression of MMR proteins is inefficient (not correlated with G₂ + M phase fraction) and is dissociated (only one gene product correlates with G₂ + M), which would eventually result in mutation accumulation and progression^[41].

INTRATUMOR HETEROGENEITY AND MICROSATELLITE ANALYSIS

Tumor cell heterogeneity is linked to genetic instability and biologic progression. This problem must be studied by including several tumor samples of sufficient size from each tumor.

The sample size is an important parameter. Microdissection techniques allow selectively picking up very small samples, which can show false cellular homogeneity, based on the loss of heterozygosity or allelic imbalance. If the tumor cell populations selected for molecular analysis are taken before they become a biologically prominent component (with kinetic or invasive advantages), the results might be confusing and clinically non-relevant. This would be a case of tumor microheterogeneity, which tends to give disparate results with meanings essentially unknown. Except for intraepithelial proliferation, all

microdissected cell samples provide target cell-rich samples with varying degrees of host cell contamination (including stromal, inflammatory, and endothelial cells). Therefore, multiple samples from the same case should always be studied and assays performed in duplicate before accepting the results as relevant.

The intratumor heterogeneity can result in discordant results for a given marker depending on the sample origin. The comparison of MMR protein expression and PCR-based MSI studies has revealed discordant results in 8% of right-sided sporadic CRC and complete concordance after performing further analyses on other tumor areas^[43]. Because of this intratumor heterogeneity, at least two samples from each CRC should be screened, although no systematic approach has been used to address this topic in sporadic CRC. Microsatellite analysis in muscle-invasive transitional cell carcinomas of the bladder have revealed topographic heterogeneity in 32% of cases, showing that the deep compartment had more microsatellite abnormalities (20%)^[25]. We have found significant differences between superficial (tumor cells above the muscularis propria) and the deep (tumor cells infiltrating the muscularis propria) compartments of sporadic CRC, the deep compartments showing MMR protein down-regulation and increased MSI^[41,44]. At least one-third of unstable tumors in deep compartments can be expected to be stable in superficial compartments. These differences can eventually result in the classification of a given tumor as MMS or MSI depending on the sample origin (superficial or deep).

CLINICAL TESTING FOR MMR DEFECTS

MSI results from the dysfunction of MMR proteins, which can be detected at genetic or protein levels. It is recommended that a CRC should be tested for MSI prior to gene testing, since this test is inexpensive and will help predict whether or not an individual has a germline MMR gene mutation^[45,46]. Since up to 5% of HNPCC tumors do not have MSI, negative MSI tests cannot completely rule out HNPCC. Conversely, a positive MSI test is not diagnostic of HNPCC because 15%-30% of unselected CRC have MSI (due to *MLH1* promoter methylation), whereas only 1%-6% of all CRC are associated with detectable HNPCC mutations. If the tumor is MSI-positive, further analyses for MMR defects are recommended.

Genetic testing for MMR defects

MMR defects are due to either inactivating point mutations spread throughout the genes, therefore needing full-length sequencing, or promoter hypermethylation (especially *MLH1* in sporadic CRC). HNPCC is an autosomal dominant disorder caused by germline MMR gene mutations, in particular in *MLH1*, *MSH2*, *MSH6*, and *PMS2*. No strong genotype-phenotype correlations have been observed to date, but *MSH2* mutations do appear to be associated with more extracolonic manifestations than *MLH1* mutations. *MSH6* mutations are more common in endometrial tumors and *PMS2* mutations are especially

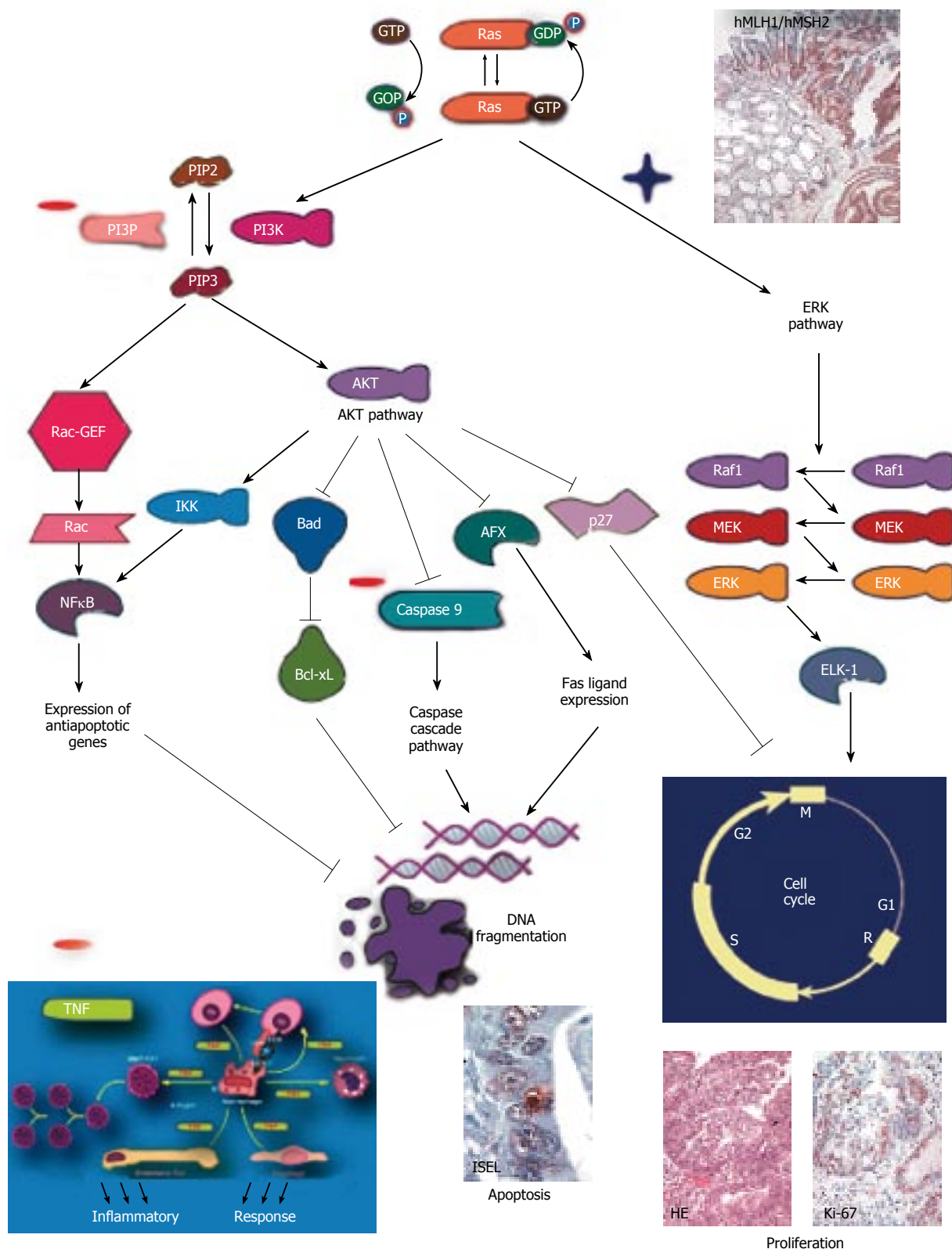


Figure 2 Molecular pathways contributing to the phenotype of microsatellite stable (MSS) CRC. MMR protein expression results in enhanced RAS signaling through ERK pathway (increased proliferation) and down-regulation of PI3P phosphatase, caspase 9 (apoptosis blockade), as well as TNF (decreased inflammation).

associated with Turcot's syndrome^[47]. The original HNPCC diagnostic criteria were established by the International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer (ICG-HNPCC) and are known as the Amsterdam criteria^[30], but only 50%-70% of HNPCC

families meeting these criteria have been found to have germline *MSH2* or *MLH1* mutations^[48]. The Amsterdam criteria were revised by the ICG-HNPCC in 1999 to include extracolonic cancers. The least stringent criteria are the Bethesda guidelines (more sensitive but less specific

than either the Amsterdam I or Amsterdam II criteria in identifying HNPCC families with pathogenic mutations), which aim to determine which patients should have MSI testing^[48]. These criteria propose MSI testing for:

Individuals with cancer in families that meet the Amsterdam criteria.

Individuals with two hereditary nonpolyposis colon cancer syndrome (HNPCC)-related cancers, including synchronous and metachronous colorectal cancers or associated extracolonic cancers (endometrial, ovarian, gastric, hepatobiliary, or small bowel cancer or transitional cell carcinoma of the renal pelvis or ureter).

Individuals with colorectal cancer and a first-degree relative with colorectal cancer and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma: one of the cancers diagnosed by age 45, and the adenoma diagnosed by age 40.

Individuals with colorectal cancer or endometrial cancer diagnosed by age 45.

Individuals with right-sided colorectal cancer with an undifferentiated pattern (solid/cribriform) on histopathology diagnosed by age 45.

Individuals with signet-ring-cell-type colorectal cancer diagnosed by age 45.

Individuals with adenomas diagnosed by age 40.

The American Gastroenterological Association recommends genetic testing for HNPCC for individuals from families meeting Amsterdam criteria, as well as for individuals with two HNPCC-related cancers (for instance, colorectal and endometrial cancer) and individuals with colorectal cancer who have a first degree relative with an HNPCC-related cancer (or colorectal adenoma) where at least one was diagnosed before age 50^[45,46,49]. Ideally, testing should first be offered to a family member with colorectal or endometrial cancer^[26,45,48,49]. In some individuals, genetic analysis may be offered after prescreening for MSI in an HNPCC-related tumor specimen. Such prescreening should be offered where an HNPCC-related cancer is present in two individuals related by first-degree regardless of age of onset, or individuals with early-onset CRC regardless of family history. Genetic testing is indicated if MSI is present.

The majority (90%) of mutation-positive HNPCC cases are caused by mutations in *MLH1* or *MSH2*^[1,9]. For this reason, the mutation analysis is generally performed for these two genes, *MSH6* being included in the analysis more recently. Although several methods can be used to detect these mutations, direct exon-by-exon gene sequencing is considered the gold standard. The sequencing should analyze each of the protein-coding regions of the *MLH1* and *MSH2* genes in their entirety, with all positive results being repeated for confirmation. Once a specific mutation that has been found in a relative by previous genetic testing, a test examining only the specific portion of the gene containing the known familial mutation can be offered to all family members.

There are some benefits and limitations of genetic testing for HNPCC. Relying solely on family history can underestimate the risk of developing cancer in mutation carriers and over-estimate risk in those who do not

inherit the mutation. When an individual has a personal or family history that suggests the possibility of HNPCC, an important step is to determine whether the person is interested in genetic testing. Genetic testing for HNPCC can have important benefits for members of high-risk families who choose to be tested^[50]. Those who are found to carry deleterious mutations can take steps to reduce their cancer risk, especially through earlier and more intensive surveillance or consideration of prophylactic surgery. Individuals with HNPCC-related CRC can undergo surgical management designed to address the increased risk of a second cancer.

In families in whom a deleterious mutation has been found, those who are mutation-negative can be spared the need for more intensive surveillance and intervention^[50]. However, these individuals remain at risk for sporadic CRC and should be encouraged to adhere to age-appropriate general population screening guidelines.

Before consenting to genetic analysis, patients should also consider the limitations of testing. Currently, genetic testing cannot detect unusual mutations responsible for HNPCC, such as those occurring in MMR genes other than *MLH1* and *MSH2*. Therefore, a negative result in an individual who does not have a family member with a documented mutation must be interpreted cautiously. The test may also detect a variant of uncertain significance whose effect on cancer risk has not yet been established. In such situations, testing other family members for the specific variant to determine if it is associated with cancer may provide clarification of the significance^[50].

Immunohistochemical testing for MMR defects

At the protein level, hMLH1/hMSH2 immunohistochemistry has a role in detecting MMR defects^[51-53], with data suggesting that the effectiveness of immunohistochemical screening of the MMR proteins would be similar to that of the more complex strategy of microsatellite genotyping^[54]. This technique can guide which gene to sequence and can help differentiating sporadic from hereditary mutations: hMSH2 loss is likely to be HNPCC, whereas hMLH1 loss could be HNPCC or sporadic CRC (*MLH1* promoter methylation). MMR proteins heterodimerize to function; the hMSH2 loss almost always accompanies hMSH6 loss and when hMLH1 is lost, generally so is hPMS2^[55-57]. In addition, immunohistochemistry can miss functional loss; i.e. presence of the protein with antigen positivity in the absence of function. Several antibodies have been used for these analyses, but the most widely used are hMSH2 (clone FE11, Oncogene Research), hMLH1 (clones G168 728 and G168-15, BD Pharmingen), hMSH6 (clone 44, BD Transduction Laboratories), and hPMS2 (clone A16-4, BD Pharmingen, and polyclonal C terminus, Santa Cruz Biotechnology).

MMR immunohistochemical studies are based on a complete absence of at least one MMR protein^[5,12,37,51-53,58-61]. But these studies do not consider the immunostaining topographic heterogeneity^[41]. Since the MMR proteins function as heterodimers, it could be advocated to validate the immunohistochemical results of hMSH2/hMSH6 and

hMLH1/hPMS2. More studies are required to clarify the influence of this predictable tumor heterogeneity to select the appropriate sample for immunohistochemical and/or MSI analyses.

PROGNOSTIC AND THERAPEUTIC IMPLICATIONS OF MSI

The CRC microsatellite profile provides useful prognostic information^[6,26,39], showing the patients with microsatellite-unstable neoplasms have a better overall survival rate and a modified response to conventional chemotherapy^[62-67]. MSI also helps in predicting the treatment response of CRC^[63,64,68], and could modify the chemotherapy protocols offered to the patients in the future^[64], but these results should be applied with caution before this predictive tool is verified^[64].

Molecular markers as predictive factors in treatment decisions have been developed in the last few years. The initial studies in sporadic CRC showed that the retention of heterozygosity at one or more 17p or 18q alleles in microsatellite-stable CRCs and mutation of the gene for the type II receptor for TGF- β 1 in CRCs with high levels of microsatellite instability correlated with a favorable outcome after adjuvant chemotherapy with fluorouracil-based regimens, especially for stage III CRC^[63,68]. However, most recent studies have revealed that fluorouracil-based adjuvant chemotherapy benefited patients with stage II or stage III CRC with MSS tumors or tumors exhibiting low-frequency MSI but not those with CRCs exhibiting high-frequency MSI^[64]. The reasons for these responses must be related to the distinctive cell kinetics associated with MMR down-regulation (significantly increased apoptosis and decreased proliferation), which can certainly contribute to tumor cell resistance to conventional chemotherapy^[40,41]. The topographic heterogeneity of sporadic CRC is a key element to explain the discrepant results reported^[41]. This point has not been systematically addressed yet, but a homogeneous selection of the samples from the same topography must be considered in the molecular test design^[25].

CONCLUSIONS

Many CRC show MSI, for which confirmatory analyses are warranted because of prognostic and therapeutic implications. Pathologists play a critical role in identifying microsatellite-unstable CRC, such as occur in young patients with synchronous or metachronous tumors and tumors with classic histologic features. In these cases, MSI testing and/or MMR immunohistochemistry are advisable, along with sequencing and genetic counseling if appropriate. Microsatellite analysis is an excellent functional and prognostic test, whereas MMR immunohistochemistry can guide gene sequencing but can result in false negatives (false negative in antigen positive neoplasms, especially cases with MLH1 promoter methylation). Direct exon-by-exon gene sequencing is considered the gold standard and should be used to analyze each of the protein-coding regions of the *MLH1* and *MSH2* genes in their

entirety, although this technique will miss *MLH1* gene inactivation by promoter methylation. Finally, the selection of samples for molecular tests must be carefully designed considering predictable heterogeneity, such as topographic heterogeneity, to avoid misinterpretations.

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Probiotics and prebiotics in chronic inflammatory bowel diseases

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Abstract

The prokaryotic and eukaryotic cells of the colon exist in a highly complex, but harmonious relationship. Disturbances in this remarkable symbiosis can result in the development of inflammatory bowel diseases (IBD). Although the etiology of IBD is not entirely understood, it is known that the chronic inflammation of Crohn's disease, ulcerative colitis and chronic pouchitis are a result of an overly aggressive immune response to the commensal intestinal flora in genetically susceptible hosts. Recent studies have enhanced our ability to understand the interaction between the host and its intestinal microflora and the role the microflora plays in maintaining intestinal homeostasis. As we begin to understand the benefits conferred to the intestine by the microflora, the notion of modifying the composition of the bacterial load to improve human health has arisen. A significant body of research now exists investigating the role of probiotics and prebiotics in ameliorating chronic intestinal inflammation. This article will begin with an overview of the role of the commensal microflora in maintaining mucosal immune homeostasis, and how a dysregulated immune response to the intestinal microflora results in IBD. This will be followed by a summary of the use of probiotics and prebiotics in experimental and human IBD.

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Key words: Colitis; Crohn's disease; Microflora; Immunity; Probiotics; Prebiotics

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INTRODUCTION

At birth, the gastrointestinal tract is a sterile environment. Initial exposure of the gut to microbes occurs during the birthing process from the maternal fecal and vaginal flora. Within a few months after birth, a relatively stable microbial population is established^[1]. This abundant, diverse and dynamic intestinal microflora normally lives in a complex, symbiotic relationship with the eukaryotic cells of the mucosa. About 100 trillion bacterial cells benefit from the constant nutrient flow, stable temperature and niches for various metabolic requirements provided by the intestinal environment. Likewise, the host benefits from the ability of the intestinal microflora to synthesize vitamin K, exert trophic effects on intestinal epithelial cells, salvage energy from unabsorbed food by producing short-chain fatty acids, inhibit the growth of pathogens, sustain intestinal barrier integrity and maintain mucosal immune homeostasis. Studies from germ-free animals reveal that the absence of resident intestinal microflora results in significant alterations in intestinal structure and function, including slender villi, shallow crypts, low leukocyte count^[2,3], a decrease in the number and density of Peyer's patches^[4] and decreased stimulation of migrating motor complexes^[5].

In their co-evolution with bacteria, vertebrates develop pattern-recognition receptors, which are activated by specific molecular patterns unique to bacteria, fungi and viruses that are absent in eukaryotes (lipopolysaccharides, peptidoglycan, ssRNA, muramyl dipeptide, flagellins, etc). These include the Toll-like receptors (TLRs) and nucleotide oligomerization domains (NODs). TLRs and NODs are critical for the initiation of innate immune defense responses. Activation of their signaling cascades usually results in the production of pro-inflammatory cytokines. TLR signaling also provides a link between innate and adaptive immunity, as TLR signaling results in the maturation of dendritic cells, which activate adaptive immune responses^[6]. Although stimulation of these receptors in most parts of the immune system results in production of inflammatory cytokines, these ligands are not only tolerated by the gut mucosal immune system, but also essential for adaptation to intestinal bacteria and maintenance of homeostasis^[7]. The tolerance to the intestinal microflora is not completely understood, but several aspects of commensal physiology have been defined which contribute to their inability to activate the immune system. Some commensal bacteria can modify

TLR ligands, resulting in a hypoactive immune response. For example, the endotoxic portion of LPS is pentacyclated in many *Bacteroides* species, and has minimal toxicity^[8]. An important feature of commensal bacteria is their inability to penetrate the intestinal epithelial barrier. If some of these organisms do penetrate, they are usually rapidly phagocytosed by the innate mucosal immune system. Indeed, in a healthy host, the systemic immune system appears to be ignorant of the intestinal microflora^[9]. Maintaining tolerance to these intestinal bacteria is a remarkable accomplishment achieved by the mucosal immune system, and disturbances in this bacterial-epithelial homeostasis result in considerable deleterious effects on the host.

Role of the commensal flora in IBD

Although many studies have investigated the possibility of a single infectious agent causing Crohn's disease and ulcerative colitis, also called chronic inflammatory bowel disease (IBD), none has yet been discovered. The intestinal bacteria are now believed to be involved in the initiation and perpetuation of IBD. The prevailing theory explaining the development of IBD is that the adaptive immune system is hyper-responsive to the commensal intestinal microflora in genetically susceptible individuals^[10]. This hypothesis is supported by several observations: most inflammation occurs in areas with the highest density of intestinal bacteria, broad spectrum antibiotics improve chronic intestinal inflammation, and surgical diversion of the fecal stream can prevent recurrence of Crohn's disease. Most importantly, despite differences in the pathogenesis of chronic intestinal inflammation, a consistent feature of many animal models of IBD (such as IL-10 knockout mice and HLA-B27 transgenic rats) is the failure to develop chronic intestinal inflammation when these animals are raised in germ-free conditions^[11,12]. Dysbiosis is also observed in IBD patients. Adherent and intramucosal bacteria, particularly *Bacteroides* spp, *Escherichia coli* (*E. coli*) and *Enterobacterium* spp are more abundant in patients with Crohn's disease (CD) than in controls^[13,14] (Figure 1). Bacterial overgrowth and dysbiosis are also associated with the development of chronic pouchitis, the inflammation of the ileal reservoir created after ileo-anal anastomosis following colectomy in ulcerative colitis (UC) patients^[15]. In addition, several selected commensal bacterial species can induce and perpetuate colitis in genetically susceptible rodent models of chronic intestinal inflammation^[16,17] (Figure 1).

Other immune dysfunctions in IBD include aberrant secretion of pro-inflammatory cytokines and chemokines by intestinal epithelial cells and mucosal immune cells^[18-20], altered TLR4 signaling^[21], defective antigen presenting cell function^[22] and lack of T-cell apoptosis^[23]. It is now clear that ignorance of the systemic immune system to commensal intestinal microflora is lost in IBD patients, as shown by enhanced and persistent cell-mediated and humoral immune responses to these bacteria^[24].

At least 7 genetic loci conferring susceptibility to CD, ulcerative colitis (UC) or both, have been identified^[25]. Interestingly, such susceptibility genes associated with CD

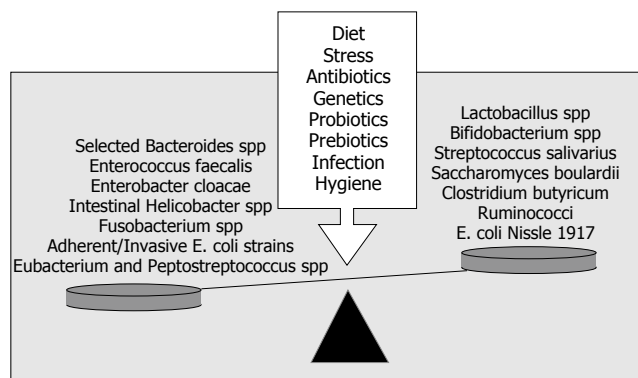


Figure 1 Microbial balance and dysbiosis. The pathogenic immune responses present in IBD are triggered by the presence of luminal bacteria. The balance of beneficial vs aggressive intestinal microbes is responsible for either mucosal homeostasis or chronic inflammation. A number of environmental and genetic factors influence the balance of beneficial vs aggressive microbes. Adapted from^[63].

involve polymorphisms of the NOD2 gene^[26] (the pattern recognition receptor for muramyl dipeptide) which can result in hampered innate immune functions by impairment of TLR function^[27], defective clearance of invasive bacteria by macrophages^[28], and decreased production of defensins^[29].

The recognition of the compelling association between intestinal microflora and the development of IBD has led to an abundance of studies investigating the therapeutic potential of altering luminal bacteria using probiotics and/or prebiotics.

Probiotics, prebiotics and synbiotics

Probiotics are defined as living organisms in food and dietary supplements which, upon ingestion, improve the health of the host beyond their inherent basic nutrition^[30]. Probiotics are typically lactic acid bacteria selected from the gut flora, and are able to survive stomach acid and bile, maintain viability throughout extended periods of storage and are safe for human consumption. Other species have also shown some beneficial effects, such as *E. coli* Nissle 1917 and *Saccharomyces boulardii* (Figure 1). Probiotic bacteria have verifiable beneficial properties, including the ability to improve epithelial barrier function, modulate the mucosal immune system, and alter the intestinal flora.

Prebiotics are non-digestible dietary carbohydrates, e.g., lactosucrose, fructo- and galacto-oligosaccharides, inulin, psyllium, bran, germinated barley (Figure 2), which stimulate the growth and metabolism of endogenous enteric protective bacteria upon consumption. Beneficial effects of prebiotics are also associated with changes in colonic short-chain fatty acids (SCFA) due to fermentation by colonic bacteria^[31]. Synbiotics are combinations of probiotics and prebiotics, and are also an emerging therapeutic modality. Restoration of normal microflora using probiotics, prebiotics or synbiotics has been investigated in numerous gastrointestinal and other disease states, including infectious diarrhea, *H. pylori* infection, irritable bowel syndrome, colorectal cancer, lactase deficiency, pancreatitis, atopy, and IBD.

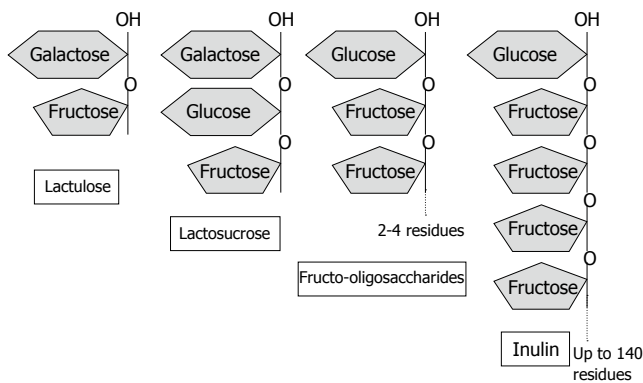


Figure 2 Basic structures of various prebiotic substances. Structurally, prebiotics are a mixture of polymers and oligomers comprising branching chains of fructose units.

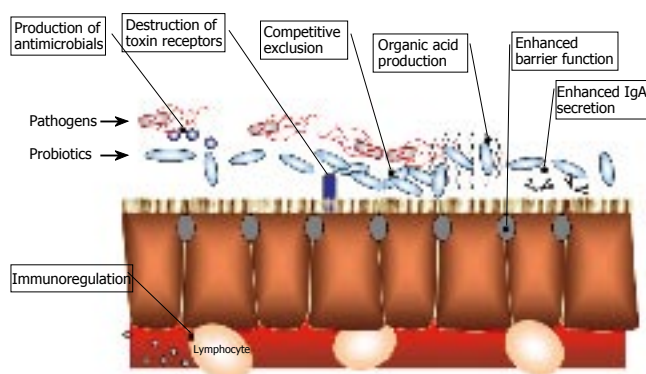


Figure 3 Mechanisms of probiotic activity.

Protective mechanisms of probiotics by ameliorating chronic intestinal inflammation

Probiotic bacteria have beneficial effects on the intestinal epithelia both directly and indirectly, including enhanced barrier function, modulation of the mucosal immune system, production of antimicrobials, and alteration of the intestinal microflora (Figure 3).

Alteration of the mucosal immune system. The presence of probiotics has been shown to result in several modifications in the mucosal immune response, including augmented antibody production^[32,33], increased phagocyte^[34] and natural killer cell activity^[35-38], modulation of the nuclear factor kappa-B (NFκB) pathway^[39-41], and induction of T cell apoptosis^[42]. Generally, probiotics increase the production of intestinal anti-inflammatory cytokines (such as IL-10 and TGF-β), while reducing the production of pro-inflammatory cytokines (e.g., TNF-α, interferon-γ, IL-8)^[43-46]. Several probiotic bacteria, including *B. breve*, *Streptococcus thermophilus*, *B. bifidum* and *Ruminococcus gnavus* have been shown to secrete metabolites that reduce LPS-induced TNF-α secretion^[47]. *L. reuteri* reduces TNF-α and *Salmonella typhimurium* induces IL-8 secretion *in vitro*, by inhibiting nuclear translocation of NFκB and preventing the degradation of IκB^[48]. Administration of the probiotic cocktail VSL#3 (consisting of *L. acidophilus*, *L. bulgaricus*, *L. casei*, *L. plantarum*, *B. breve*, *B. infantis*, *B. longum*, *S. thermophilus*) to IL-10 deficient mice results in

colitis reduction and a concomitant reduction in mucosal secretion of TNF-α and interferon-γ^[49]. *E. coli* Nissle 1917 is able to down-regulate the expansion of newly recruited T-cells into the mucosa and limit chronic intestinal inflammation^[50]. In SAMP1/Yit mice, *Lactobacillus casei* strain Shirota inhibits IL-6 production in LPS-stimulated large intestinal lamina propria mononuclear cells and down-regulates nuclear translocation of NFκB^[51]. Patients with a recent ileo-anal pouch anastomosis who responded to probiotic therapy have reduced mRNA levels of IL-1β, IL-8 and IFN-γ, and fewer polymorphonuclear cells compared with patients who receive placebo^[52]. Probiotic treatment has also been shown to reduce IFN-γ and IL1-α expression and decrease inducible-nitric oxide synthase and gelatinase activities in pouch biopsy samples from patients with pouchitis^[53]. In mucosal explants of ileal specimens from patients with Crohn's disease, probiotics reduced TNF-α release and the number of CD4 cells^[54]. In addition to live probiotics, components of probiotic bacteria can also exert effects on the mucosal immune system. For example, genomic DNA isolated from VSL#3 inhibits TNF-α-induced IL-8 secretion, mitogen-activated protein kinase activation and NFκB activation^[41] in HT-29 cells.

Improved barrier function. Various probiotic bacteria can enhance intestinal epithelial barrier function. For example, oral administration of VSL#3 results in normalization of impaired colonic barrier function and restoration of intestinal epithelial integrity in IL-10 deficient mice and enhancement of epithelial resistance in T-84 cells^[49]. Barrier function was enhanced not only by live bacteria, but also by a proteinaceous secreted product of VSL#3^[49]. Several strains of lactobacilli are also capable of up-regulating intestinal MUC3 mRNA expression, thereby improving barrier function by increasing the mucus layer^[55,56]. *Lactobacillus* GG (*L. GG*) improves barrier function by inhibiting apoptosis of intestinal epithelial cells^[57]. *S. thermophilus* and *L. acidophilus* have been shown to enhance phosphorylation of actinin and occludin in the tight junction, thereby preventing the invasion of enteroinvasive *E. coli* into human intestinal epithelial cells^[58].

Alteration of the intestinal flora. Probiotics suppress the growth and invasion of pathogens in several ways. They competitively exclude pathogenic bacteria by occupying the limited physical space in the mucus layer and on epithelial cells. They also engage pattern-recognition receptors and consume substrate otherwise available to other (pathogenic) microbes. In addition, probiotics render their microenvironment inauspicious for pathogens by secreting antimicrobial substances such as hydrogen peroxide, organic acids, and bacteriocins. For example, both *in vitro* and *in vivo* experiments demonstrate that *B. infantis* suppresses the growth of *Bacteroides vulgatus*^[59]. VSL#3 has been shown to inhibit *Salmonella dublin* invasion into T-84 cells^[49]. Patients with pouchitis treated with VSL#3 have been demonstrated to have increased bacterial diversity in the pouch, and decreased fungal diversity^[60].

Probiotics may also alter the intestinal microflora by changing the fatty acid profile in the colon. VSL#3

Table 1 Clinical studies of probiotics in IBD

Author	Design	Group (Dose/d) (n)		Results
		Probiotic	Comparator	
Induction of remission of ulcerative colitis				
Kato 2004 ^[87]	DB, R, C	Bifidobacterium fermented milk (100 mL) (10)	Placebo (10)	Reduced UCDAI (<i>P</i> < 0.05)
Rembacken 1999 ^[85]	DB, R, C	<i>E. coli</i> Nissle 1917 (1 × 10 ¹¹ cfu) (57)	Mesalamine (59)	As effective as mesalamine at attaining remission
Bibiloni 2005 ^[88]	Open-label	VSL#3 (3.6 × 10 ⁹ cfu) (32)	None	77% remission or response rate
Ishikawa 2003 ^[86]	R, C	Lactobacillus and Bifidobacterium-fermented milk (100 mL) (11)	Placebo (10)	Reduced exacerbation of symptoms (<i>P</i> < 0.01)
Borody 2003 ^[90]	Case reports	Fecal enema (6)	None	100% remission
Maintenance of remission of ulcerative colitis				
Kruis 2004 ^[82]	DB, R, C	<i>E. coli</i> Nissle 1917 (2.5-25 × 10 ⁹ cfu) (162)	Mesalamine (165)	As effective as mesalamine at maintaining remission (<i>P</i> = 0.003)
Zocco 2006 ^[83]	Open-label	Lactobacillus GG (1.8 × 10 ¹⁰ cfu) (65)	Mesalamine (60) Mesalamine + LGG (62)	No difference in relapse rates at 12 mo. LGG more effective than mesalamine at prolonging relapse-free time (<i>P</i> < 0.05)
Shanahan 2006 ^[117]	DB, R, C	Lactobacillus salivarius or Bifidobacterium infantus (1 × 10 ⁹ cfu) (52/group)	Placebo (53)	No improvement of time to relapse
Venturi 1999 ^[84]	Open-label	VSL#3 (1 × 10 ¹² cfu) (20)	None	75% maintained clinical and endoscopic remission
Induction of remission of Crohn's disease				
Schultz 2004 ^[97]	DB, R, C	Lactobacillus GG (2 × 10 ⁹ cfu) (5)	Placebo (6)	No difference in remission rates
McCarthy 2001 ^[118]	Open-label	Lactobacillus salivarius (1 × 10 ¹⁰ cfu) (25)	None	Reduced disease activity compared with baseline
Gupta 2000 ^[119]	Open-label	Lactobacillus GG (2 × 10 ¹⁰ cfu) (4)	None	Improvement in CDAI scores compared with baseline (<i>P</i> < 0.05)
Maintenance of remission of Crohn's disease				
Prantera 2002 ^[95]	DB, R, C	Lactobacillus GG (1.2 × 10 ¹⁰ cfu) (23)	Placebo (22)	No significant difference in remission
Campieri 2000 ^[120]	R, C	VSL#3 (3 × 10 ¹¹ cfu) (20)	Mesalamine (20)	Equivalent to mesalamine in preventing recurrence
Marteau 2006 ^[94]	DB, R, C	Lactobacillus johnsonii LA1 (2 × 10 ⁹ cfu) (48)	Placebo (50)	No difference in endoscopic recurrence
Malchow 1997 ^[93]	DB, R, C	<i>E. coli</i> Nissle 1917 (5 × 10 ¹⁰ cfu) (16)	Placebo (12)	No difference in remission rates
Bousvaros 2005 ^[96]	DB, R, C	Lactobacillus GG (2 × 10 ¹⁰ cfu) (39)	Placebo (36)	No difference in time to relapse
Guslandi 2000 ^[91]	R, C 6 mo	Saccharomyces boulardii (1 g/d) + mesalamine (2g) (16)	Mesalamine (16)	Significant prolongation of remission (<i>P</i> < 0.05)
Induction of remission of pouchitis				
Kuisma 2003 ^[80]	DB, R, C	Lactobacillus GG (1 × 10 ¹⁰ cfu) (10)	Placebo (10)	No difference in PDAI
Laake 2004 ^[121]	Open-label	Lactobacillus acidophilus and Bifidobacterium lactis-fermented milk (500 mL) (51)	None	Improved PDAI, no difference in histology
Gionchetti 2000 ^[78]	DB, R, C	VSL#3 (6 g) (20)	Placebo (20)	Increased remission time (<i>P</i> < 0.001)
Mimura 2004 ^[122]	DB, R, C	VSL#3 (6 g) (20)	Placebo (16)	Increased remission time (<i>P</i> < 0.0001)
Gionchetti 2003 ^[79]	DB, R, C	VSL#3 (1 × 10 ¹¹) (20)	Placebo (20)	Increased remission time (<i>P</i> < 0.05)

DB: Double-blind; R: Randomized; C: Controlled; UCDAI: Ulcerative colitis disease activity index; CDAI: Crohn's disease activity index; LGG: Lactobacillus GG; PDAI: Pouchitis disease activity index.

probiotic strains are also capable of converting linoleic acid to conjugated linoleic acid, a fatty acid with anti-inflammatory and anti-carcinogenic properties^[61].

Use of probiotics in inflammatory bowel disease treatment

Results from various animal studies and clinical trials using probiotics to treat intestinal inflammation have generated considerable excitement. Data are now emerging which suggest that probiotics are capable of preventing relapse of chronic intestinal inflammation. Some probiotics can even treat mild to moderately active IBD^[62,63]. However,

at present there is a relative lack of rigorously designed, randomized, placebo-controlled trials. Level 1 evidence is only available for the use of probiotics in post-operative chronic pouchitis while level 2 and 3 evidence supports the use of probiotics in treatment of CD and UC^[62] (Table 1).

Experimental colitis

More than 20 animal models of IBD are available^[64] and have been widely used to study the efficacy and mechanisms of probiotics in ameliorating inflammation in order to provide support for human clinical trials. In

IL-10 knockout mice, *L. plantarum* 299v^[65], *L. reuteri*^[46], *L. salivarius* subspecies *salivarius* 433118, *B. infantis* 35624^[66], *L. salivarius* subspecies *salivarius* UCC118^[67] and VSL#3^[49] have all been shown to successfully attenuate intestinal inflammation. *L. GG* prevents recurrent colitis in HLA-B27 transgenic rats after antibiotic treatment, whereas *L. plantarum* has no effect^[68]. Both VSL#3 and *L. GG* significantly ameliorate sulphydryl-blocker iodoacetamide-induced colitis in rats, whereas they have no effect on dinitrobenzene sulfonic acid-induced colitis^[69]. Improved inflammation in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis has also been demonstrated after oral administration of *L. salivarius* ssp. *salivarius* CECT5713 and *L. plantarum* NCIMB8826^[70,71]. Dextran sulphate sodium (DSS)-induced colitis in mice is ameliorated by soluble bacterial antigens extracted from *E. coli* (strain *Laves*) or by *Bifidobacterium* strains *breve*, *catenulatum*, and *longum*^[72,73]. Daily administration of live but not heat-killed auto-aggregating *L. crispatus* reduces the severity of DSS-colitis in mice^[74]. *L. reuteri* significantly reduces the colonic inflammation caused by both acetic acid and methotrexate in rats^[75,76]. Interestingly, DNA from VSL#3 has been reported to reduce colonic inflammation, thus improving intestinal barrier function in IL-10 KO mice and DSS-induced colitis^[41,77].

Chronic pouchitis

Probiotics can maintain antibiotic-induced remission in patients with chronic pouchitis after colectomy for refractory UC. Gionchetti *et al.*^[78] have completed placebo-controlled trials using the probiotic cocktail VSL#3 in patients with chronic relapsing pouchitis, showing that the remission rate after 1 year is 90% in the group treated with VSL#3 versus 60% in the placebo group. Another study by the same group also showed that VSL#3 is also capable of preventing the development of chronic pouchitis during the first year after pouch surgery for UC^[79]. Ten percent of patients who took VSL#3 developed pouchitis, compared with 40% of the placebo group. A double-blinded, prospective, randomized placebo-controlled trial was carried out in 20 patients treated with *L. GG* versus placebo for 3-mo^[80]. In contrast to the study with the probiotic cocktail VSL#3, no significant differences were observed in chronic pouchitis disease activity in *L. GG*-treated patients despite an increased total fecal lactobacilli: anaerobe ratio, illustrating the inefficacy of *L. GG* for this condition.

Ulcerative colitis

Numerous studies have investigated the use of probiotics for maintenance of remission of UC in humans. A small study investigating the use of non-pathogenic *E. coli* Nissle 1917 versus low-dose mesalamine showed that it can maintain remission of quiescent UC, with a relapse rate of 16%-67% in those treated with *E. coli* versus 11%-73% in the mesalamine group^[81]. In a double-blind, randomized trial involving 327 patients, Kruis *et al.*^[82] compared the effectiveness of an oral preparation of *E. coli* Nissle 1917 with mesalamine for maintaining remission of UC, and found that at the end of the 12-mo study, there is no significant difference between the two study groups, with

relapses occurring in 36.4% of the *E. coli* Nissle 1917 group and 33.9% of the mesalamine group (significant equivalence, $P = 0.003$). Recently, Zocco *et al.*^[83] investigated the efficacy of *L. GG* in maintaining remission of UC and found that there is no difference in relapse rate between the 3 groups after 6 and 12 mo. However, *L. GG* is more effective than standard mesalamine treatment in prolonging relapse-free time ($P < 0.05$). An open-label study of VSL#3 showed that 15/20 UC patients remain in remission after 1 year^[84].

Several studies have addressed treatment of established UC with probiotic therapy. A study using *E. coli* Nissle 1917 has demonstrated its equivalence to mesalamine for inducing remission of UC^[85]. Ishikawa *et al.*^[86] evaluated *Bifidobacterium*-fermented milk in the treatment of UC, and symptoms of exacerbation were observed in 3 of 11 patients in the treated group versus 9 of 10 patients in the untreated group after 1 year ($P = 0.01$). However, no difference was observed in endoscopic disease activity. A placebo-controlled trial with bifidobacteria-fermented milk for 12 wk in UC patients with active disease showed that endoscopic disease activity index and histological score are significantly reduced in the treatment group compared with those in placebo group^[87]. Uncontrolled administration of daily VSL#3 for 3 mo induces remission in 19 of 30 patients (63%), with a response rate of 87%^[88]. As in all of these reported studies, the increased luminal probiotic bacteria return to baseline levels within one month after stopping probiotic treatment, indicating only transient colonization by these probiotic bacteria. In another small study administration of a combination of 3 bifidobacterium species for 2 mo was superior (20% remission) to placebo (93% remission) in maintaining remission of UC induced by sulfasalazine and glucocorticoids^[89]. This effect correlates with decreased mucosal TNF- α , IL-1 β and increased mucosal IL-10 levels^[89]. An interesting study by Borody *et al.*^[90] showed that altering the gut microflora in UC patients achieved dramatic outcomes by administration of a freshly prepared enema from a healthy donor to six patients with relapsing refractory UC after broad spectrum antibiotics. This results in a remarkable reversal of all symptoms after 4 mo and sustained remission after 1-3 year of treatment.

Crohn's disease

Thus far, the use of probiotics for the prevention and treatment of CD is less substantiated than for the prevention and treatment of UC, although some studies certainly show promise. The effect of probiotics in maintaining remission of CD has been reported in an open-label study in patients receiving mesalamine alone versus mesalamine and *S. boulardii*^[91]. At 6 mo, 37.5% of patients had a clinical relapse in the former group versus 6.3% of patients in the probiotic group. In an open-labeled study, McCarthy *et al.*^[92] reported that oral administration of *L. salivarius* UCC118 significantly reduces disease activity in patients with mild to moderate CD. In a randomized, placebo-controlled pilot study, patients with CD were treated with steroids and randomized to non-

Table 2 Clinical studies of prebiotics in IBD

Group (Dose/d) (n)				
Author	Design	Prebiotic	Comparator	Results
Induction of remission of ulcerative colitis				
Kanauchi 2003 ^[112]	Open label	Conventional therapy + Germinated barley foodstuff (20-30 g) (21)	Conventional therapy	Improved UCDAI
Furrie 2005 ^[114]	DB, R, C	B. longum (2 × 10 ¹¹) + inulin/ oligofructose (6 g) (9)	Placebo (9)	Sigmoidoscopy scores reduced (P = 0.06) β-defensins, TNFα , IL-1α levels decreased (P < 0.05)
Maintenance of remission of ulcerative colitis				
Hanai 2004 ^[113]	Open label	Conventional therapy + Germinated barley foodstuff (20 g) (22)	Conventional therapy (37)	Improved UCDAI and endoscopic scores
Induction of remission of Crohn’s disease				
Lindsay 2006 ^[115]	Open label	Fructo-oligosaccharides (15 g) (10)	None	Increase in IL-10 expressing intestinal dendritic cells

pathogenic *E. coli* Nissle 1917 as probiotic therapy or placebo^[93]. After 1 year, there were fewer relapses in the probiotic group, but this was not statistically significant. Despite these promising studies, there are numerous reports on the inefficacy of some probiotics in CD. A randomized, placebo-controlled study of 98 patients showed that *L. johnsonii* LA1 is ineffective in preventing postoperative recurrence of CD^[94]. A placebo-controlled trial with *L. GG* is also ineffective in preventing postoperative recurrence of CD in patients undergoing bowel resection^[95]. Other studies have failed to detect benefits of *L. GG* in maintaining remission of CD in children^[96] and adults^[97,98]. Although *L. GG* has demonstrated its efficacy in treating rotaviral^[99] and antibiotic-associated^[100] diarrhea, results in IBD patients have been particularly underwhelming for this bacterial species, highlighting the species-specificity of colitis protection by probiotics.

PREBIOTICS IN INFLAMMATORY BOWEL DISEASE

Experimental colitis

Studies using prebiotics have been performed mostly in animal models. Lactulose and inulin have been shown to attenuate inflammation in IL-10 knockout mice and DSS-induced colitis respectively^[46,101]. The combination of inulin and oligofructose (mixture 1:1) is also effective in preventing the development of colitis in HLA-B27 transgenic rats^[102]. This beneficial effect is observed in conjunction with an increase of intestinal bifidobacteria and lactobacilli. Another study in HLA-B27 transgenic rats showed that the effects of the synbiotic "SIM", a combination of lactobacilli, bifidobacteria and the prebiotic inulin, are attributed to the inulin rather than the probiotics. The ingested probiotic bacteria are not detectable in the cecal content, yet the microflora profile of their cecal contents is altered^[103]. In that study, inulin was also shown to specifically stimulate the growth of *Bifidobacterium animalis*. DSS-induced colitis rats fed with goat's milk oligosaccharides maintain their body weight, have reduced colonic myeloperoxidase activity and clinical

symptoms and increased MUC-3 expression compared with control rats^[104]. Goat's milk oligosaccharides also causes decreased anorexia, weight loss, bowel wall thickening and necrotic lesions in TNBS-induced colitis in rats, compared with untreated controls^[105]. In another study of TNBS-induced colitis in rats, a 2-wk feeding of lactulose prior to the induction of colitis reduces myeloperoxidase activity, colonic TNF α and leukotriene B production, in conjunction with an increase of lactobacilli and bifidobacteria species in feces^[106]. Lactulose has also demonstrated a dose-dependent beneficial effect on DSS-induced colitis in rats, including improvements of colonic ulceration areas, body weight changes, diarrhea, bloody stools and a reduction of myeloperoxidase activity and microscopic colitis^[107].

However, not all studies using prebiotics have resulted in positive outcomes. Moreau *et al*^[108] found that fructo-oligosaccharides are ineffective in improving DSS-induced colitis in rats. Holma *et al*^[109] have reported a similar inefficacy of galacto-oligosaccharides in TNBS-colitis rats.

Several studies have investigated the use of an insoluble mixture of glutamine-rich protein and hemicellulose-rich dietary fiber termed germinated barley foodstuff (GBF). Fukuda *et al*^[110] found that feeding GBF to rats with DSS-induced colitis results in significantly reduced colonic inflammation scores, and increased butyrate concentrations in cecal contents. Kanauchi *et al*^[111] have observed similar results in DSS-colitis rats, and further determined that the dietary fiber component rather than the protein component of the GBF is responsible for the beneficial effects of GBF.

Ulcerative colitis

Table 2 lists the clinical trials using prebiotics to treat IBD. Although there is a paucity of human studies using prebiotics, the few emerging studies showed that there is potential for this treatment modality. A multi-centered open-label trial reported that oral administration of GBF to patients with mild to moderately active UC for 24 wk results in a significant decrease in clinical activity index, compared to controls^[112]. An open-label study of 22 UC

patients in remission showed that a daily oral intake of 20 g GBF results in a significantly improved clinical activity index and endoscopic score at 3, 6 and 12 mo, and a reduced relapse rate, compared with controls^[113]. A recent randomized, double-blinded controlled trial by Furrie *et al.*^[114] examined the use of synbiotics in 18 patients with active UC, using a combination therapy of *B. longum*, inulin and oligofructose, and found that sigmoidoscopy inflammation scores are reduced in the synbiotic-treated population when compared to placebo. Intestinal levels of TNF- α and IL-1 β are also reduced. Additionally, rectal biopsies have demonstrated reduced inflammation and greater epithelial regeneration in the synbiotic-treatment group.

Crohn's disease

A small, uncontrolled study of 15 active CD patients reported that 21 d of fructo-oligosaccharide (15 g) intake results in a significant decrease of disease activity, an increase of intestinal bifidobacteria and modifications of Toll-like receptors and IL-10 expression in mucosal dendritic cells^[115].

CONCLUSION

The link between intestinal microflora and IBD is now well established, and altering the composition of the microflora using probiotics and prebiotics holds promise as a therapeutic strategy for ameliorating chronic intestinal inflammation. Future developments in this field must include rigorous double-blind, placebo-controlled trials, using probiotics and prebiotics along with a further understanding of their protective mechanisms. Due to their excellent safety profile and lack of serious side effects, there are few contraindications to the consumption of prebiotics, probiotics and synbiotics by IBD patients. Further understanding of the interactions between microbes and gastrointestinal tract will help identify which strains of bacteria and/or which prebiotics may be effective in the treatment of different types of chronic inflammatory disease.

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Delayed gastric emptying is associated with pylorus-preserving but not classical Whipple pancreaticoduodenectomy: A review of the literature and critical reappraisal of the implicated pathomechanism

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Abstract

Pylorus-preserving pancreaticoduodenectomy (PPPD) is nowadays considered the treatment of choice for periampullary tumors, namely carcinoma of the head, neck, or uncinate process of the pancreas, the ampulla of Vater, distal common bile duct or carcinoma of the peri-Vaterian duodenum. Delayed gastric emptying (DGE) comprises one of the most troublesome complications of this procedure. A search of the literature using Pubmed/Medline was performed to identify clinical trials examining the incidence rate of DGE following standard Whipple pancreaticoduodenectomy (PD) vs PPPD. Additionally we performed a thorough in-depth analysis of the implicated pathomechanism underlying the occurrence of DGE after PPPD. In contrast to early studies, the majority of recently performed clinical trials demonstrated no significant association between the occurrence of DGE with either PD or PPPD. PD and PPPD procedures are equally effective operations regarding the postoperative occurrence of DGE. Further randomized trials are required to investigate the efficacy of a recently reported (but not yet tested in large-scale studies) modification, that is, PPPD with antecolic duodenojejunostomy.

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Key words: Pylorus-preserving pancreaticoduodenectomy; Whipple pancreaticoduodenectomy; Delayed gastric emptying; Pancreatic surgery

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INTRODUCTION

The introduction of partial pancreaticoduodenectomy for the treatment of carcinoma of the ampulla of Vater dates back to almost a century ago and is credited to Kausch, a German surgeon from Berlin^[1]. Then, in 1935 Whipple and associates redefined this procedure as a two-stage pancreaticoduodenectomy, where the pylorus and proximal duodenum are closed and preserved, while gastrointestinal continuity is re-established via a gastrojejunostomy^[2]. Six years later, the first successful one-stage radical pancreaticoduodenectomy in which the distal stomach, pylorus and duodenum are removed, was reported independently by Whipple^[3] and Trimble and coworkers^[4]. Whipple is credited with popularizing the procedure, which now bears his name. Whipple pancreaticoduodenectomy (PD) has become the standard procedure of choice for many decades for the treatment of benign disorders requiring pancreaticoduodenectomy (such as chronic pancreatitis)^[5], as well as for the treatment of periampullary tumors (carcinoma of the head, neck, or uncinate process of the pancreas, ampulla of the Vater, distal common bile duct, or peri-Vaterian duodenum)^[6,7]. In 1978, Traverso and Longmire^[8] reported a technique by which the whole stomach and 2.5 cm of the duodenum are preserved, restoring the gastrointestinal continuity by duodenojejunostomy. By application of pylorus-preserving pancreaticoduodenectomy (PPPD), the postgastrectomy syndrome (postprandial dumping, diarrhea, dyspepsia, nausea and vomiting) following Whipple resection is reduced and better functional results are achieved^[9]. Although this technique has been initially reported by Watson^[10] more than three decades before, the study by Traverso and Longmire^[8] did not receive enough attention and has not been widely applied. In recent years, PPPD has been used increasingly by many surgeons, and is

considered the treatment of choice in many pancreatic surgery reference centers worldwide, despite the opinion that PPPD does not allow adequate resection of pancreatic or periampullary tumors^[11,12].

A shorter operating time and a reduced intraoperative blood loss as a result of omission of gastric resection requiring the transfusion of fewer units of blood, as well as avoidance of PD-related dumping syndrome, better postoperative weight gain and a better quality of life, are considered advantages of PPPD over PD^[8,13-20]. However, PPPD has been linked with a major drawback, that is, delayed gastric emptying (DGE), which is responsible for prolonged hospital stay and increased associated morbidity^[11,21-23]. DGE has been reported in early studies to occur in up to 70% of patients undergoing PPPD procedure^[11,14,15,22-28]. Although the incidence of DGE appears to be declining in later published reports^[5,29-31], DGE remains a leading cause of PPPD postoperative complications. The concern regarding an increased incidence of DGE following PPPD has prevented the adoption of this technique by some major American pancreatic centers^[32].

Due to the fact that no uniform definition for DGE following pancreatic surgery exists, numerous controversial opinions have been reported regarding the efficacy of these techniques as causative factors for postoperative DGE occurrence. In an attempt to demonstrate which procedure, PD or PPPD, is preferable with regards to post-operative DGE occurrence, we performed a review of randomized, controlled trials in the English literature investigating the incidence of DGE following PPPD compared with PD. We further analyzed the various implicated pathomechanisms leading to the occurrence of DGE.

DEFINITION OF DGE

The occurrence of DGE following PPPD is initially reported by Warsaw and Torchiana^[22]. In their study that included 8 patients undergoing PPPD, only 1 tolerated solid food within 10 postoperative days. Early studies have used a wide variety of definitions for DGE following pancreatic surgery. Some researchers have defined DGE as the inability to tolerate a regular or normal diet by the tenth^[14,22,27] or fourteenth^[26] postoperative day, or the start of a liquid diet after ≥ 7 d^[25]. Others have described DGE as gastric stasis requiring gastric suction for 7 d^[11,15] or ≥ 10 d^[24,28].

In the recent years, although various definitions for DGE exist, 3 seem to be most widely acceptable. (1) According to the first definition^[24], DGE occurs when the nasogastric tube is left in place for ≥ 10 d plus one of the following: emesis after removal of the nasogastric tube, reinsertion of a nasogastric tube, postoperative use of prokinetic agents after the 10th postoperative day, or failure to progress with diet. (2) According to the second definition^[33], DGE occurs when nasogastric intubation is required ≥ 10 d following the operation, or is reinserted due to vomiting. (3) According to the third definition^[34], DGE occurs when nasogastric intubation is required ≥ 10 d following the procedure or when a solid diet cannot be tolerated on or before the 14th postoperative day.

Independent of the correct definition, DGE not only leads to repeated episodes of nausea and vomiting, but also has an impact on postoperative weight gain, duration of hospitalization^[26] and related morbidity, while it may also lead to fatal aspiration and pneumonia^[35]. It is therefore a dangerous and potentially life-threatening complication.

WHAT IS THE PATHOMECHANISM UNDERLYING THE OCCURRENCE OF DGE?

A number of theories have been postulated to explain the occurrence of DGE after PPPD. Physiological gastric emptying and motility of the digestive system are complex processes that are controlled and regulated by complicated physiological mechanisms. Tonic contractions of the proximal stomach are important for the transfer of liquid food from the stomach to the duodenum^[36,37], while peristaltic contractions of the distal stomach are of primary importance for reducing the size of the solid food particles and for the transfer of solid food to the duodenum^[38]. Furthermore, certain properties of ingested food, such as volume, osmolality, pH and nutrient content, may down-regulate the motility of the digestive system, either via vagal and splanchnic sensory pathways which mediate inhibition of gastric motility induced by duodenal distension^[39], or *via* cholecystokin- and secretin-mediated pathways^[40].

Cholecystokinin (CKK) has been shown in animals and humans to inhibit gastric emptying (especially the liquid-phase emptying of the stomach)^[41] *via* a vagal capsaicin-sensitive afferent pathway and by stimulating phasic and tonic pyloric motility^[42-45]. Muller and associates^[46] reported that CCK levels decrease from 1.1 ± 0.2 pmol/L preoperatively to 0.8 ± 0.2 pmol/L 10 d postoperatively, and to 0.5 ± 0.1 pmol/L following PPPD, though no statistical significance could be demonstrated. The decrease in CCK levels is attributed to the resection of the duodenum, because high concentrations of CCK are found in the duodenal mucosa.

An alternative explanation is that the reduction of CCK levels is an adaptive response to DGE. Large amounts of CCK can also be released from the jejunum, as proven by the fact that bypassing of the duodenum in patients with Billroth II gastrectomy does not decrease CCK secretion after ingestion of fats^[47]. A finding that supports this theory is that blockage of CCK receptors with antagonists accelerates gastric emptying^[48].

The role of plasma secretin levels in the development of DGE following PPPD remains controversial and has not yet been fully elucidated^[46,49-50]. Another mechanism that has been demonstrated to influence food transit is CCK-mediated pancreatic polypeptide (PP) release, which is mainly controlled by vagal cholinergic mechanisms^[51-53]. Studies in dogs and humans have shown that the duodenum and vagal innervation are necessary for normal postprandial release of PP from pancreas^[54,55]. PPPD has been shown to be significantly associated with reduction of PP levels compared with the preoperative findings^[46]. This reduction seems to be due to resection of the pancreatic head, where the majority of PP-producing cells

are located^[54].

Other researchers support the theory that DGE occurs as a direct result of the removal of the duodenum, which influences gastric secretion and emptying as well as pancreatic and biliary secretion, thus playing an important role in the regulation of pancreatic hormone release^[56,57]. In addition, duodenectomy disrupts the coordination of gastric and intestinal migrating motor complexes^[58], decreases the postprandial PP release^[59], and abolishes the interdigestive cycles of plasma PP^[54]. Other investigators believe that preservation of the duodenal pacemaker located 0.5-1 cm distally from the pylorus should be the mainstay of the procedure, in order to avoid disturbances in normal gastric peristalsis^[60]. Gastric dysrhythmias probably exacerbated by some intra-abdominal complications such as an anastomotic leak or an abscess have also been thought to be the causative factor for DGE following PPPD^[61]. In addition, problems caused by the surgical procedure itself, namely the injury to the nerve of Latarjet, or placement of suture material through the pyloric muscle resulting in ischemia of the gastroduodenal segments and gastroparesis, have similarly been implicated^[15].

Multiple other causative agents have been implicated as etiological factors for DGE after PPPD, namely intra-abdominal complications, such as a leakage or an abscess^[16,26,61,62], postoperative pancreatitis^[63], pancreatic fibrosis^[64], preoperative cholangitis^[62], pylorospasm secondary to vagal injuries that requires the performance of pyloromyotomy^[65], alternation of the endocrinologic milieu^[15,19], early enteral nutrition commencing on the first postoperative day^[66], and torsion or angulation of the reconstructed alimentary tract^[23,67]. It has been advocated that preservation of the right gastric artery is essential for avoidance of DGE, because of its arterial supply to the pylorus and antrum^[23], although there are other investigators who do not support this theory^[64]. A more recent experimental study suggests that division of neurovascular supply to the pylorus and/or transection of the duodenum may lead to DGE following PPPD^[68]. These investigators underlined that, besides the right gastric artery, additional preservation of the supraduodenal artery, as well as conservation of the pyloric branch of the vagus nerve, are crucial for avoidance of DGE following PPPD.

In an interesting study from two surgical institutes in the Netherlands^[69], the choice of Billroth I (proximal end-to-end duodenojejunostomy) or Billroth II (end-to-side pancreatojejunostomy at the end of the jejunal loop, followed by end-to-side hepaticojejunostomy and an end-to-side duodenojejunostomy) type of reconstruction has been shown to influence DGE after PPPD. Although significantly less procedure-related complications were noted following Billroth I compared to Billroth II type of reconstruction (18% *vs* 42% respectively, $P < 0.05$), DGE occurred in significantly more patients receiving Billroth I compared to Billroth II type of reconstruction (76% *vs* 32% respectively, $P < 0.05$).

The type of reconstruction of pancreaticogastrointestinal continuity following pancreatoduodenectomy has also been implicated to play a significant role in the

development of DGE. A randomized study comparing pancreaticogastrostomy [PG] (69 patients) *vs* end-to-side pancreatojejunostomy [PJ] (82 patients)^[70], showed that PG is superior regarding DGE rates (2 *vs* 10 patients, or 3% *vs* 12%, respectively, $P = 0.03$). By using the PG reconstructive technique (a single layer of nonabsorbable interrupted stitches on the posterior wall of the stomach)^[71] instead of PJ (single layer pancreatojejunal or duct to mucosa technique)^[72], the authors found that significantly less complications occur (25% *vs* 68%, respectively, $P = 0.002$). More specifically, the lower rates of biliary fistulae (0% *vs* 8.5%, respectively, $P = 0.01$) and intra-abdominal fluid collections (10% *vs* 27%, respectively, $P = 0.01$) following PG compared with PJ, are the main culprits for the decreased rates of DGE.

Postoperative complications have been reported to correlate significantly with the occurrence of DGE in other trials as well. Horstmann and associates^[73] showed that the incidence of DGE increases from 1% when no postoperative complications occur, to 28% and 43% respectively when moderate (wound infection, temporary cardiopulmonary complications, transient occurrence of amylase/lipase-rich drainage fluid without signs of sepsis) and severe (anastomotic leakage, bleeding, septic complications, reoperation) complications occur ($P < 0.0001$). The results of several other studies lend support to this theory^[16,27,33,74-77].

An in-depth analysis of the physiology of the mechanism underlying the occurrence of DGE has also been reported, showing that the initiation of interdigestive phase III is closely related to the elevation of plasma motilin concentration^[78]. Motilin, a 22-aminoacid residue polypeptide, originates in motilin cells, which are scattered in the duodenal epithelium^[78]. Erythromycin and related 14-member macrolide compounds act as motilin agonists by binding to motilin receptors, which are largely confined to the antrum of the stomach and the upper duodenum^[79], thus initiating phase 3 activity of the interdigestive migratory motor complex (MMC)^[80-81]. An early study^[24] showed that patients administering high doses (200 mg) of erythromycin every 6 h from postoperative d 3 to 10 have a 53% reduction in the incidence of DGE compared with placebo.

Studies in unfed normal patients have shown that high doses of erythromycin (200-300 mg) induce strong, prolonged bursts of antral contraction, which are not propagated to the small intestine^[82,83]. On the contrary, erythromycin administered in low doses (40 mg) induces premature phase 3, commencing in the stomach and migrating through the small intestine, which is similar to spontaneously occurring phase 3^[82]. To test this hypothesis, Ohwada and coworkers^[84] performed a prospective randomized, placebo-controlled trial investigating the effect of low-dose erythromycin *vs* placebo administration on DGE following PPPD and demonstrated that intravenous administration of erythromycin lactobionate (1 mg/kg) in 50 mL of 0.9% saline, given over 15 min through a central venous route every 8 h from postoperative d 1 to 14 results in reduction in the incidence of DGE following PPPD compared with placebo (14.3% *vs* 57.1% for erythromycin and placebo respectively, $P = 0.04$). Use of low-dose

erythromycin is significantly associated with induction of phase 3 of the MMC and initiation of phase 3-like contractions ($P < 0.0001$), earlier nasogastric tube removal ($P < 0.001$) and earlier progression to diet ($P < 0.003$). In contrast, the number of patients who had a nasogastric tube reinserted and emesis after nasogastric tube removal was similar in both groups. Still, erythromycin administration was associated with a 75% reduction in the incidence of DGE. In addition, a stepwise multiple regression analysis using a Cox proportional hazard model, showed that erythromycin and preservation of right gastric artery are significant covariates. Right gastric artery removal is a predictive factor for the effectiveness of erythromycin. The authors concluded that a low dose of erythromycin is not only more effective in reducing DGE after PPPD, but is also associated with a much lower rate of adverse effects compared with a high dose.

Octreotide, a long-lasting somatostatin analogue^[85], administered preoperatively and continued postoperatively for 7 d at a dosage of 100 µg given subcutaneously 3 times a day has been reported to accelerate the rate of gastric emptying^[86]. A randomized, placebo controlled trial in healthy volunteers^[87], showed that administration of octreotide in the above-mentioned dosage can significantly accelerate gastric emptying compared to placebo ($P < 0.05$). It is hypothesized that this occurs as a result of the suppression of postprandial CCK release. A role in the prevention of DGE following pancreatic surgery is thus suggested. A randomized, placebo-controlled report^[88], however, has questioned the role of octreotide in pancreaticoduodenectomy procedures. A similar study^[89] showed that although octreotide use is associated with decreased rates of DGE compared with non-use, its use is significantly associated with the development of pancreatic fistulae. Based on their findings, the authors suggest avoidance of routine use of octreotide after pancreaticoduodenectomies until the development of international guidelines.

IS DGE SIGNIFICANTLY ASSOCIATED WITH PPPD, BUT NOT WITH PD?

We searched the Medline/Pubmed database for clinical studies comparing the efficacy of PD *versus* PPPD with regards to DGE excluding publications not in the English language (Table 1). As a result, a total of 17 trials investigating the incidence of DGE after PPPD compared with PD are identified^[6,12,20,25,34,63,66,73,77,90-97]. On the whole, 910 patients undergoing PD are compared with 1078 patients undergoing PPPD. Therefore, a total of 1988 patients have participated in these 17 studies.

Most early studies^[12,25,63,90] showed that PD is superior to PPPD regarding incidence rates of DGE. However, only one study has demonstrated statistical significance in this outcome^[25]. Another study showed that the difference in the occurrence rates of DGE after the two procedures is not significant because DGE when presents, resolves spontaneously within 6 wk. Later performed studies seem to support that the incidence rates of DGE following either PD or PPPD are comparable^[34,63,66,73,77,91,93,95], although supporters of PD over PPPD regarding DGE

rates also exist^[94]. Some recent trials have even provided significantly lower rates of DGE following PPPD than following PD^[92,96,97].

The reasons behind this diversity are multifactorial. The definition of DGE following pancreatic surgery varies from study to study. Improvement of surgical technique and increased surgical experience as well as advances in perioperative and critical care management, have resulted in decreased rates of DGE in recent years. The degree of lymph node dissection and pancreatic resection as well as the performance of anastomoses vary in different centers. Peri-operative administration of drugs that have been shown to decrease post-operational rates of DGE, like octreotide or erythromycin lactobionate, varies from study to study. The indication for performing PD varies significantly not only between different studies, but also within the same patient cohort. There is therefore a growing need for a multicentre, randomized clinical trial with specific guidelines for peri-operative administration of pharmaceutical agents, standard definition of the term DGE, and specific etiology-based performance of pancreatic surgery, to compare the efficiency of the two methods regarding DGE.

DISCUSSION

A recently reported modification in the classical PPPD procedure is the performance of duodenojejunostomy antecolically instead of retrocolically. Traverso and Kozuschek^[98] reported a decade ago that antecolic duodenojejunostomy seems to be preferred by an increasing number of pancreatic surgery centers worldwide^[61,69,99-101]. The theoretical background for this technique is that decreased blood circulation (especially venous drainage) of the jejunal limb following biliary-pancreato-enteric reconstructions can lead to decreased motility and profound edema of the jejunal limb itself, and eventually edema of the duodenojejunal anastomosis^[27]. Compromised venous drainage of the jejunal limb, which is the peristalsis starting point of the newly constructed intestinal pathway, might lead to delayed recovery of jejunal peristalsis at the site of duodenojejunostomy, which will then cause DGE^[62]. From a theoretical point of view, antecolic duodenojejunostomy avoids mechanical problems, because the descending jejunal loop is more mobile than after retrocolic reconstruction.

Kurosaki and Hatakeyama^[99] evaluated the results of antecolic duodenojejunostomy in 55 consecutive patients undergoing PPPD as the selected mode of therapy for a wide variety of underlying diseases, and demonstrated that by use of the antecolic jejunal reconstruction method, DGE is markedly reduced based on the choice of the definition of DGE selected. According to the definition by Fabre *et al*^[33], DGE occurs in only 5.5% patients. According to the definition by van Berge Henegouwen *et al*^[34], DGE occurs in 29.1% patients, while according to the definition by Yeo *et al*^[24], DGE occurs in 18.2% patients.

These researchers demonstrated that the development of a major complication is correlated significantly with reinsertion of nasogastric tube or emesis ($P = 0.010$), a later initiation of liquid diet ($P = 0.0381$) and a later

Table 1 Association between DGE and PD/PPPD

Study	Yr	Patients (n)	Results
Klinkenbijn <i>et al</i> ^[20]	1992	91 (44 PDs, 47 PPPDs)	No difference with regards to DGE was demonstrated between the two groups (i.e. days to liquid and normal diet)
Roder <i>et al</i> ^[12]	1992	110 (62 PDs, 48 PPPDs)	DGE was noted in 0 (0%) patients after PD and 9 (19%) patients after PPPD (<i>P</i> value not mentioned)
Patel <i>et al</i> ^[25]	1995	67 (52 PDs, 15 PPPDs)	DGE was noted in 41% of the PD group and 61% of the PPPD group (<i>P</i> = 0.04)
Mosca <i>et al</i> ^[90]	1997	218 (61 PDs, 157 PPPDs)	DGE was noted in 1 (4.7%) patient after PD and 14 (8.9%) patients after PPPD (<i>P</i> value not mentioned).
van Berge Henegouwen <i>et al</i> ^[34]	1997	200 (100 PDs, 100 PPPDs)	DGE was noted in 34 patients after PD and 37 patients after PPPD (<i>P</i> = NS) ¹
Lin and Lin ^[63]	1999	30 (15 PDs, 15 PPPDs)	DGE was noted in 1 patient after PD and 6 patients after PPPD (<i>P</i> = 0.08, two-sided Fisher's exact test, NS)
Di Carlo <i>et al</i> ^[91]	1999	113 (39 PDs, 74 PPPDs)	DGE was noted in 6 (15.3%) patients after PD and 9 (12.1%) patients after PPPD (<i>P</i> = NS)
Yeo <i>et al</i> ^[92]	1999	106 (58 PDs, 48 PPPDs) ²	DGE was noted in 9 (16%) patients after PD and 2 (4%) patients after PPPD (<i>P</i> = 0.03)
Seiler <i>et al</i> ^[93]	2000	77 (40 PDs, 37 PPPDs)	DGE was noted in 18 (45%) patients after PD and 12 (32%) patients after PPPD (<i>P</i> = 0.17, NS)
Martignoni <i>et al</i> ^[66]	2000	62 (27 PDs, 35 PPPDs)	DGE was noted in 9 (33%) patients after PD and 13 (37%) patients after PPPD (<i>P</i> = NS)
Yamaguchi <i>et al</i> ^[94]	2001	50 (27 PDs, 23 PPPDs)	DGE was significantly associated with PPPD compared with PD (gastric tube removal, <i>P</i> < 0.0001, oral intake, <i>P</i> = 0.0018)
Yeo <i>et al</i> ^[6]	2002	294 (148 PDs, 146 PPPDs)	DGE was noted in 24 (16%) patients after PD and 9 (6%) patients after PPPD (<i>P</i> = 0.006)
Nguyen <i>et al</i> ^[95]	2003	105 (50 PDs, 55 PPPDs) ³	DGE was noted in 6 of 50 (12%) patients after PD and 4 of 55 (7%) patients after PPPD (<i>P</i> = 0.40, NS)
Horstmann <i>et al</i> ^[73]	2004	132 (19 PDs, 113 PPPDs) ⁴	DGE was noted in 4 of 19 (21%) patients after PD and 13 of 113 (12%) patients after PPPD (<i>P</i> = 0.11, NS)
Tran <i>et al</i> ^[77]	2004	170 (83 PDs, 87 PPPDs) ⁵	DGE was noted in 18 patients after PD and 19 patients after PPPD (<i>P</i> = 0.80, NS)
Seiler <i>et al</i> ^[96]	2005	130 (66 PDs, 64 PPPDs)	DGE was noted in 30 (45%) patients after PD and 20 (31%) patients after PPPD (<i>P</i> = 0.096, NS)
Lin <i>et al</i> ^[97]	2005	33 (19 PDs, 14 PPPDs) ⁶	DGE was noted in 6 (43%) patients after PD and 0 patients after PPPD (<i>P</i> < 0.05)

NS: Not significant. ¹Although nasogastric intubation was prolonged after PPPD *vs* PD (3 *vs* 6 d, *P* < 0.0001), this did not influence DGE rates; ²Initially 114 patients were included in the study. Of these, 58 underwent PD while the remaining 56 were scheduled for PPPD. However, in 8 patients, the pylorus could not be preserved. They were therefore not included in the results; ³In 7 of 55 (13%) patients in the PPPD group, the pylorus could not be preserved; ⁴A total of 150 patients were included in the study but the 18 patients that underwent duodenum-preserving pancreatic head resection were not included here; ⁵Two patients in the PPPD group were converted to the PD group during operation as the surgeon expected duodenal involvement; ⁶Initially 36 patients were included in the study. Three patients with pancreatic head adenocarcinoma initially assigned to the PPPD group, had to undergo PD eventually due to extensive duodenal involvement. These 3 patients were not calculated in either study groups.

progression to solid diet (*P* = 0.0343). Furthermore, a major complication is correlated significantly with DGE but only according to the definition of DGE by Yeo *et al*^[24] (*P* = 0.0006), and not according to the definition of DGE by Fabre *et al*^[33] (*P* = 0.421) or van Berge Henegouwen *et al*^[34] (*P* = 0.103). A major complication is defined as a condition requiring invasive treatment or intensive care, or a pancreatic fistula proved by amylase-rich (> 1000 mg/dL) fluid from drains over 7 postoperative days or radiological examination. In their group, 10 patients developed a major complication (five patients required intensive care or invasive treatment, while another 5 developed a pancreatic fistula).

In addition, division of the left gastric vein (LGV) is correlated significantly with the occurrence of DGE (5.3% *vs* 37%, if the LGV is preserved or divided, respectively, *P* = 0.0016) according to the definition of DGE by van Berge Henegouwen *et al*^[34] but not according to the definition of DGE by Yeo *et al*^[24] (0% *vs* 5%, if the LGV is preserved or divided, respectively, *P* = 0.067). After summing up their results, the authors concluded that, by

setting the stomach vertically in the left abdomen, antecolic duodenojejunostomy improves the occurrence of DGE after PPPD.

The decreased incidence of DGE following antemesenteric instead of retromesenteric jejunal reconstruction has been verified by other researchers as well. Park and associates^[62] demonstrated that antemesenteric jejunal reconstruction is associated with a significantly lower incidence of DGE compared to retromesenteric reconstruction (6.5% *vs* 31.7%, respectively, *P* < 0.05) in terms of duration and amount of nasogastric drainage, as well as diet progression. Sugiyama and associates^[100] also support the superiority of antemesenteric jejunal reconstruction with regards to DGE (8% *vs* 72% incidence of DGE for antemesenteric *vs* retromesenteric jejunal reconstruction respectively, *P* < 0.001). Horstmann and colleagues^[73] demonstrated that performance of antecolic duodenojejunostomy-PPPD is associated with reduced (though not statistically significant) rates of DGE compared with standard Whipple (12% *vs* 21% for antecolic duodenojejunostomy-PPPD and

standard Whipple procedure respectively, $P = 0.11$).

So far, a major drawback of all reported studies is the lack of randomization. Their interpretation has therefore noticeable limitations. Recently however, two randomized controlled trials have verified the positive effect of the antecolic reconstruction method on DGE rates^[102,103]. The first study^[102] reported a significantly lower incidence of DGE after antecolic compared with retrocolic duodenojejunostomy (5% vs 50% respectively, $P = 0.0014$). However, due to the small number of patients included in this study ($n = 20$ patients/group), the authors support that larger-scale studies are needed to confirm the positive results of this new reconstruction method. The second study^[103] demonstrated the same positive results (5.0% vs 24.0% for antecolic and retrocolic duodenojejunostomy, respectively, Odds Ratio: 0.167, 95% CI: 0.054-0.430, $P < 0.001$). Although the number of patients included in this trial was not as small ($n = 100$ patients/group)^[103] as in the first study^[102], a significant drawback is the difference in the time periods of sample collection (from January 1, 1996 until December 31, 2001 for the retromesenteric group, and from January 1, 2002 until December 31, 2003 for the antemesenteric group). Standardization of the operative technique, as well as continuous improvement in perioperative management, could account in part for the difference observed in DGE rates.

CONCLUSION

Pylorus-preserving pancreatic head resection and classical Whipple are equal operations regarding the postoperative development of delayed gastric emptying. Further randomized controlled trials are required to confirm the advantage of antecolic versus retrocolic duodenojejunostomy in PPPD.

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Induction of apoptosis on human hepatocarcinoma cell lines by an alkyl resorcinol isolated from *Lithraea molleoides*

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INTRODUCTION

Hepatocellular carcinoma is the 5th most common cancer in the world and the 4th most common cause of cancer-associated mortality^[1]. Surgical resection and local treatment are frequently limited, as a result of metastasis, cirrhosis, and other pathological changes in the liver parenchyma. The development of chemotherapeutic or chemopreventive agents for hepatocellular carcinoma is important to reduce the mortality caused by this disease. Since cell homeostasis depends on the balance between proliferation and apoptosis, effective compounds inducing apoptosis appear to be a relevant strategy to suppress tumor growth^[2]. Cytotoxic drugs cause cell death in sensitive cells, at least partly, by induction of apoptosis.

The Anacardiaceae family comprises many medicinal species from which a number of biologically active substances, such as various phenolic lipids (alkylresorcinols, alkylphenols, alkylcatechols), have been isolated. These compounds present antibacterial, fungicidal and cytotoxic properties^[3,4]. In addition, cytotoxic activity on tumor cells (B-16, PC-13, L-5178Y, P-388 and Hep-2) and antitumor activity against S-180 tumors in mice have been reported^[5,6]. *Lithraea molleoides* (Vell.) Engl, a member of the Anacardiaceae family, is a Southamerican tree that grows in Argentina, Brazil and Uruguay^[7,8]. We have previously reported cytotoxic activity for the methanol extract of *L. molleoides* on HepG2 cells^[9]. Further activity-guided fractionation of the dichloromethane extract has led to the isolation of a pure bioactive compound, a new cytotoxic 5-alkyl resorcinol (5-AR) derivative: 1,3-dihydroxy-5-(tridec-4',7'-dienyl) benzene^[10].

The aim of the present study was to analyze the mechanism of cytotoxicity of this compound, by studying apoptosis induction on treated HepG2 and Hep3B hepatoma cell lines.

Abstract

AIM: To study the mechanism of cytotoxicity of a new active 5-alkyl resorcinol [1, 3-dihydroxy-5- (tridec-4', 7'-dienyl) benzene] isolated from *Lithraea molleoides* leaves on liver tumor cells.

METHODS: Human hepatocarcinoma cell lines (HepG2 and Hep3B) in culture were treated with inhibitory concentrations, 50% of the compound, for 24 h. The induction of apoptosis was detected in treated cells by analysis of DNA fragmentation, DNA content, and acridine orange and propidium iodide staining.

RESULTS: After 24 h of 5-alkyl resorcinol treatment, both cell lines showed: (1) the typical morphological alterations of apoptosis; (2) DNA fragmentation, detected by laddering and appearance of a subG0 population by flow cytometry; and (3) condensed and fragmented nuclei by acridine orange-propidium iodide staining.

CONCLUSION: Based on the results, this compound exerts its cytotoxic effect in both hepatocellular cell lines through apoptotic cell death. For Hep3B, cells with mutated p53 and Fas, apoptosis would proceed by p53- or Fas-independent pathways.

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Key words: *Lithraea molleoides*; Cytotoxic activity; Alkyl resorcinol; Apoptosis; Hepatoma

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MATERIALS AND METHODS

Isolation and identification of the compound

The isolation and characterization of the *L. molleoides*

compound 1, 3-dihydroxy-5- (tridec-4', 7'-dienyl) benzene has been previously described^[10].

Human tumoral cell lines

HepG2 and Hep3B cells, derived from human hepatoma, were obtained from the American Type Tissue Collection (ATCC, HB 8065 and HB 8064, respectively). They were cultured in minimal Eagle's medium (MEM) supplemented with 100 ml/L fetal bovine serum (FBS), 2 mmol/L glutamine, 1.5 g/L sodium bicarbonate, 1.0 mmol/L non-essential aminoacids, 1.0 mmol/L sodium pyruvate, and incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Cytotoxicity assay (MTT assay)

The cytotoxicity assay was carried out as previously described^[9]. Briefly, 5×10^3 of HepG2 or Hep3B cells in growth medium were seeded in each well of a 96 well-microtiter plate, and incubated for 24 h at 37°C. Different concentrations of the 5-AR (in quadruplicates) were added to the exponentially growing cells. Cell controls in absence of the compound were included. After an incubation period of 24 h at 37°C, the MTT assay was performed following the manufacturer's instructions. The absorbance values at 546 nm and 650 nm were recorded in an ELISA plate reader (Meterech 960). The 50% inhibitory concentrations (IC₅₀) for both cell lines were determined by linear regression from dose-response curves.

Analysis of DNA fragmentation

Approximately $1-2 \times 10^6$ of control or treated cells were harvested and washed twice with PBS. DNA was extracted by the Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's instructions. The DNA pellet was washed, air-dried, resuspended and electrophoresed on 1% agarose gel at 50 volts for 3 h. The gel was visualized under UV transilluminator and photographed.

Analysis of the DNA content by flow cytometry (hypoploid cells)

After a 24-h 5-AR treatment, control or treated cells were harvested and washed twice with 1 g/L bovine serum albumin (BSA) in PBS. They were fixed in 700 mL/L ethanol for 1 h at 4°C, washed twice with 1 g/L BSA in PBS and resuspended in the same buffer. After the addition of a DNA extraction buffer (192 mmol/L PO₄HN₂; 4 mmol/L citric acid; pH 7.8), incubation at room temperature (RT) for 5 min and centrifugation, the cells were stained with propidium iodide (Sigma, 50 µg/mL) and treated with RNase A (Sigma, 0.5 mg/mL) for 30 min at RT. Cells were analyzed on a flow cytometer (FACSCalibur, Becton Dickinson) containing an argon laser (488 nm). The red fluorescence of propidium iodide, proportional to the DNA content, was collected through a 620 ± 15 nm band pass filter. A minimum of 10000 cells per sample was collected and DNA histograms were further analyzed by the WinMDI 2.8 program.

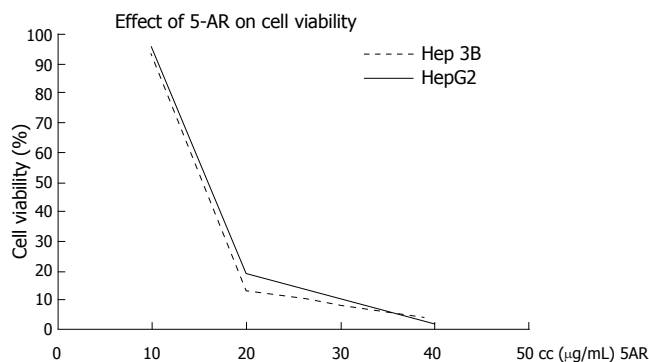


Figure 1 Treatment with 5-AR is highly cytotoxic for HepG2 and Hep3B cells. Cytotoxicity was studied testing cell viability by MTT assay in control or treated cells. The IC₅₀ values for both cell lines were determined by linear regression from dose-response curves.

Acridine orange and propidium iodide staining

Approximately 1×10^6 of control or treated cells were resuspended in 5 mL of PBS containing 5 µg/mL of acridine orange (Sigma) and 5 µg/mL of propidium iodide (Sigma). The cell suspension was immediately dispensed onto slides, viewed under fluorescent microscopy (Nikon Eclipse 400) and photographed (Nikon Coolpix 4500).

RESULTS

Cytotoxicity of 5-AR

The cytotoxicity of 5-AR on HepG2 and Hep3B human hepatoma cell lines was assessed by a cell viability assay, in the presence of different concentrations of the compound for 24 h. Under these experimental conditions, 5-AR exhibited a significant cytotoxic effect on both HepG2 and Hep3B cells. The IC₅₀ values for HepG2 and Hep3B were interpolated from linear regression curves: 13.12 µg/mL (45.49 µmol/L) and 12.45 µg/mL (43.17 µmol/L), respectively (Figure 1). Both viability curves for 5-AR were practically overlapped, suggesting a parallel effect for cell death in both cell lines. This effect is independent of the p53 status of the cells, as cytotoxicity by 5-AR was similar for HepG2, which posses wild-type p53, and Hep3B, which are p53-deficient.

Based on the detected cytotoxic activity, the effect of the compound on cell apoptosis induction was examined in both cell lines by different methods.

Effect of 5-AR treatment on cellular morphology

Apoptosis, characterized by cell shrinkage, membrane blebbing, nuclear pyknosis, chromatin condensation, and genomic fragmentation, represents a universal and exquisitely efficient cellular suicide pathway. The cytoplasm condenses and cells shrink to finally form apoptotic bodies. HepG2 or Hep3B cells were treated for 24 h with the previously determined 24 h IC₅₀ of the compound. After this period, the cells were observed under contrast phase microscopy (Nikon TMS) and photographed (Nikon FDX-35). The observation of cell morphology revealed that 5-AR-treated cells showed significant morphological

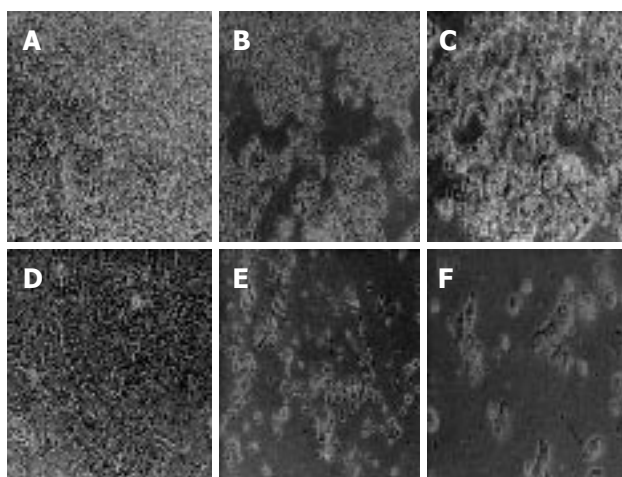


Figure 2 Treatment with 5-AR induces morphological changes typical of apoptosis in HepG2 and Hep3B cells. Control or treated cells were observed under contrast phase microscopy and photographed. **A:** Control untreated HepG2 cells (100 x magnification); **B:** HepG2 cells treated for 24 h with 5-AR IC50 (100 x magnification); **C:** HepG2 cells treated for 24 h with 5-AR IC50 (200 x magnification); **D:** control untreated Hep3B cells (100 x magnification); **E:** Hep3B cells treated for 24 h with 5-AR IC50 (100 x magnification); **F:** Hep3B cells treated for 24 h with 5-AR IC50 (200 x magnification). Results are from one experiment that is representative of three similar ones.

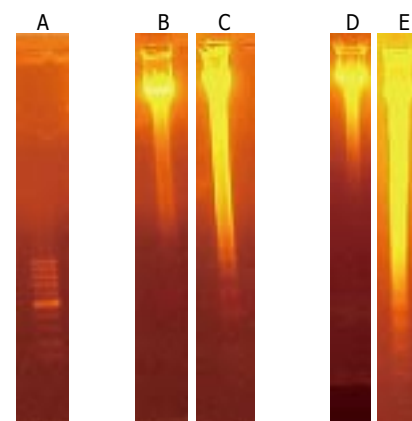


Figure 3 Treatment of HepG2 and Hep3B cells with 5-AR induces the DNA laddering typical of apoptosis. DNA was extracted from control or treated cells and electrophoresed on an agarose gel. Lane **A:** molecular marker; lane **B:** control untreated HepG2 cells; lane **C:** HepG2 cells treated for 24 h with 5-AR IC50; lane **D:** control untreated Hep3B cells; lane **E:** Hep3B cells treated for 24 h with 5-AR IC50. Results are from one experiment that is representative of three similar ones.

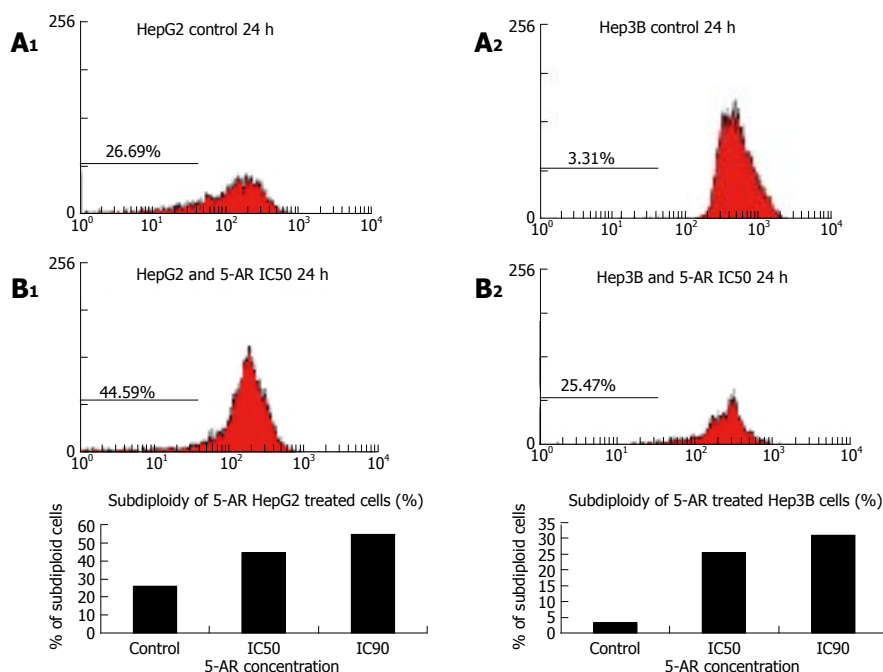


Figure 4 5-AR-treated HepG2 and Hep3B cells showing a subG0 population detected by flow cytometry. Treated or control cells were stained with propidium iodide and analyzed by flow cytometry. Left: (**A**₁) control untreated HepG2 cells, (**B**₁) HepG2 cells treated for 24 h with 5-AR IC50; Right: (**A**₂) control untreated Hep3B cells, (**B**₂) Hep3B cells treated for 24 h with 5-AR IC50. Results are from one experiment that is representative of three similar ones.

changes compatible with programmed cell death (Figure 2). The effects on cell morphology were dose-dependent (data not shown).

Effect of 5-AR treatment on DNA fragmentation

DNA fragmentation, the typical hallmark of apoptosis, was analyzed by DNA laddering on agarose gels and by the appearance of a subdiploid cell population with lower DNA content by flow cytometry.

The apoptotic process leads to fragmentation of cellular DNA in characteristic oligonucleosomal fragments, multiples of 200 base pairs. HepG2 and Hep3B cells, treated for 24 h with 5-AR, showed the typical DNA ladder on agarose gels (Figure 3). The intensity of this typical DNA ladder of multiples of 200 base pairs

fragments was dose- and incubation time-dependent.

These results were further confirmed by flow cytometer histograms obtained from treated cells, stained with propidium iodide. For HepG2 cells, the 5-AR treatment produced a significant increase in the percentage of the subG0 cell population (control cells: 26.09%; IC50: 44.59%). For Hep3B cells, the 5-AR treatment also produced a significant elevation of the subG0 cell population (control cells: 3.39%; IC50: 25.47%). The typical hypoploid population or subG0 peak, corresponding to cells with low DNA content, is shown in cytometry histograms (Figure 4).

Induction of nuclear condensation by 5-AR treatment

Control or 5-AR-treated HepG2 and Hep3B cells were

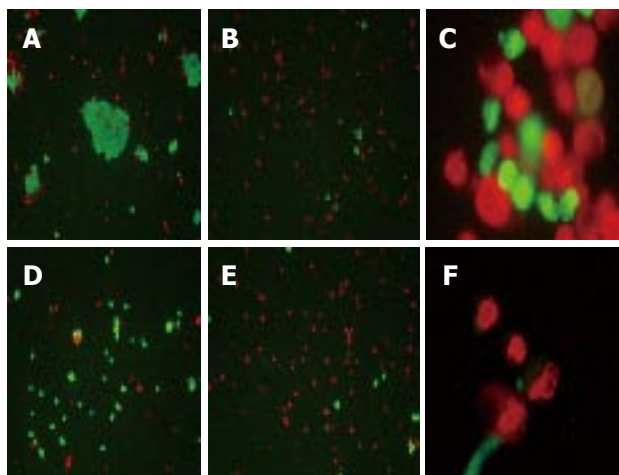


Figure 5 Treatment with 5-AR induces nuclear condensation and fragmentation in HepG2 and Hep3B cells. Cells were stained with acridine orange and propidium iodide, and observed under fluorescent microscopy. **A:** Control HepG2 cells (100 x magnification); **B:** treated HepG2 cells (100 x magnification); **C:** treated HepG2 cells (200 x magnification), showing in detail morphological changes of nuclear chromatin; **D:** control Hep3B cells (100 x magnification); **E:** treated Hep3B cells (100 x magnification); **F:** treated Hep3B cells (200 x magnification). Results are from one experiment that is representative of three similar ones.

stained with acridine orange and propidium iodide. A significant increase in the percentage of red cells and in condensed and fragmented nuclei were observed in treated cells, all these findings compatible with apoptotic cell death (Figure 5).

DISCUSSION

The search for novel anticancer drugs from natural sources has continued through the collaboration of scientists worldwide in looking for new bioactive compounds^[12]. Experimental agents derived from natural products offer opportunities to evaluate not only totally new chemical classes of anticancer agents, but also novel and potentially relevant mechanisms of action^[13].

With the aim of searching for new cytotoxic compounds from Argentine medicinal plants, a 5-AR was isolated from *L. molleoides*. The dose-dependent cytotoxic activity detected on many human tumoral cell lines suggested that it may contain some kind of antitumoral activity^[10]. Due to the relevance of human hepatocarcinoma and the lack of available successful treatments, human hepatocarcinoma cells were selected to study the 5-AR cytotoxicity and to deepen into the mechanism of cell death induced by this compound.

Apoptotic pathways are involved in the cytotoxic mechanism of antitumoral drugs. Some anticancer drugs are known to upregulate Fas ligand, leading to its interaction with Fas and triggering the apoptotic pathway. On the other hand, p53 exerts its effects on cells as a transcription factor. An increase in p53 leads to the expression of pro-apoptotic proteins, which prompt cells to undergo apoptosis. It has been reported that p53-dependent apoptosis modulates the cytotoxicity of anticancer agents^[14].

Considering the importance of Fas and p53 in hepatocyte cell death, two cell lines were selected based on

their differential p53 and Fas phenotype. While HepG2 cells exhibit normal expression of both proteins, Hep3B cells present mutated p53 and Fas^[15]. In this work, the treatment of HepG2 and Hep3B with IC50 doses of the 5-AR induced apoptosis in both cell lines, as evidenced by all the methodologies used in this work. Experiments with lower concentrations of the compounds also showed apoptosis induction in both cell lines (data not shown).

The fact that 5-AR can induce programmed cell death in Hep3B with non-functional p53^[15] and Fas^[16] evidences that the cytotoxic effect of 5-AR in this cell line is independent of their p53 or Fas phenotypic profile. As p53 is the most common mutated gene in hepatocellular carcinomas, it is important to have cytotoxic compounds that exert their apoptotic activity in a p53-independent pathway to treat this kind of tumors.

The induction of apoptosis is known to be an efficient strategy for cancer therapy^[17]. Recently, many plant extracts have demonstrated to possess the ability of triggering the apoptotic pathway^[18,19]. The present study demonstrates that a pure compound isolated from *L. molleoides* is highly cytotoxic and presents apoptogenic activity on human hepatocarcinoma cell lines. The molecular mechanistic pathway involved in this process will be studied in our future experiments. Our results and the previously reported cytotoxic and antitumoral activities of other alkyl resorcinols justify further *in vitro* and *in vivo* studies to evaluate the potential use of this 5-AR as an antitumoral agent for hepatocellular carcinoma.

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VIRAL HEPATITIS

Distribution of hepatitis B virus genotypes: Phylogenetic analysis and virological characteristics of Genotype C circulating among HBV carriers in Kolkata, Eastern India

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Abstract

AIM: To evaluate the genotype distribution of hepatitis B virus (HBV) in Eastern India and to clarify the phylogenetic origin and virological characteristics of the recently identified genotype C in this region.

METHODS: Genotype determination, T1762/A1764 mutation in the basal core promoter (BCP) and A1896 mutation in the precore region of 230 subjects were determined by restriction fragment length polymorphism method (RFLP) and the result was confirmed by direct sequencing.

RESULTS: The predominant genotypes D (HBV/D) and A (HBV/A) were detected in 131/230 (57%) and 57/230 (25%) samples. In addition, genotype C (HBV/C) was detected in 42/230 (18%) isolates. Surface gene region was sequenced from 45 isolates (27 HBV/C, 9 HBV/A and 9 HBV/D). Phylogenetic analysis revealed that all of the HBV/C sequences clustered with South East Asian subgenotype (HBV/Cs). The sequence data showed remarkable similarity with a Thai strain (AF068756) (99.5% ± 0.4% nucleotide identities) in 90% of the genotype C strains analyzed. T1762/A1764 mutation in BCP region, associated with high ALT was significantly higher in HBeAg negative isolates than HBeAg positive isolates. Frequency of A1896 mutation leading to HBeAg negativity was low.

CONCLUSION: The present study reports the genotypic distribution and the characteristics of partial genome sequences of HBV/C isolates from Eastern India. Low genetic diversity and confinement of HBV/C in Eastern India possibly indicate a recent, limited, spread in this region. Genotype C with T1762/A1764 mutation has been reported to increase the risk for hepatocellular carcinoma; therefore genotype C carriers in Eastern India should be carefully monitored.

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Key words: HBV genotypes; HBV/Cs, Eastern India; T1762/A1764 mutation

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INTRODUCTION

Hepatitis B virus (HBV) belongs to the *Hepadnaviridae* family of enveloped viruses with double-stranded DNA genome of nearly 3200 bp lengths. The HBV genome consists of four major overlapping open reading frames named surface (S), core (C), polymerase (P), and X.

HBV that infects humans has been classified into mainly eight genotypes, A-H based on the sequence divergence over the entire genome exceeding 8%^[1-4] and S gene sequence analysis^[5]. HBV genotypes have distinct geographical distributions and according to various studies seem to have different biological properties affecting, thus, the clinical outcome of HBV disease. Subgroups have been identified within different HBV genotypes, on the basis of > 4% (but < 8%) difference in the complete nucleotide sequence. In HBV genotype A, two subgroups have been defined; one is prevalent in Europe (Ae), and the other is prevalent in Africa and Asia (Aa)^[6]. Similarly, genotype C has been classified into four subgroups with characteristic

geographical distributions. Subgroup C1 (Cs) is common in Southeast Asian countries like Thailand, Myanmar and Vietnam, C2 (Ce) in East Asian countries like Japan, Korea and China^[7,8], C3 in Oceania comprising strains specifying *adrq*-, and C4 specifying *aym3* is encountered in Aborigines from Australia^[9,10].

Based on antigenic typing and further analysis of sub determinants HBV has been classified into 9 subtypes^[11]. Subtypes correlate broadly with genotypes. Some subtypes can be found in more than one genotype, which confer additional heterogeneity within the genotypes.

It is now recognized that mutations in the basal core promoter (BCP) and precore region regulate hepatitis B e antigen (HBeAg) expression. It was reported in an *in vitro* study that the double mutations in BCP A1762T and T1764A (T1762/A1764A) down regulate precore mRNA and slightly increase the efficiency of pregenome mRNA and core mRNA^[12]. Recently HBV genotypes have been partially clarified as influencing the clinical manifestation of chronic liver disease in hosts. A higher disease inducing capacity of HBV/C than HBV/B has been observed in Asia. Moreover, HBV/C with T1762/A1764 mutation has been reported to increase the risk for hepatocellular carcinoma^[13].

India is a vast country with an ethnically diverse population. With more than 40 million carriers of HBV, this is the major etiology of chronic liver diseases in India. Analyses of genomic sequences of HBV isolates from India are limited. Most reports are from Western India and Northern India, where genotypes D and A are found^[14,15]. Eastern India is a geographical area where genotypes D and A of mainland India and genotypes B and C of China and Southeast Asia converge. Recently genotype C has been reported from Eastern India^[16-18]. However, data regarding genotype distribution as well as molecular and virological characteristics of genotype C in Eastern India remain undefined.

Therefore the present study was undertaken to investigate the distribution of HBV genotypes in Eastern India and the molecular and virological features of HBV/C circulating in Eastern India.

MATERIALS AND METHODS

Patients

A cross sectional study was performed on 230 HBV DNA positive serum samples from patients with HBV infection who were referred to Indian Council of Medical Research (ICMR) Virus Unit for HBV DNA detection. Among them, 200 samples came from the outpatient clinics of Kolkata hospitals during the period of March 2001 to February 2004. All patients were known to have been positive for surface antigen (HBsAg) for > 6 mo. In addition 30 HBsAg positive asymptomatic carriers found during a community based epidemiological point prevalence study carried out by Institute of Post Graduate Medical Education & Research (IPGMER) in the rural areas, about 150 km away from Kolkata, were also included in this study^[19]. These samples were sent to our Unit for HBV DNA detection and for genotyping.

Only pretreatment samples were included in the study. Informed consent was obtained from the patients, and the Institutional Ethical Committee approved of the study protocol.

Serological testing

HBsAg, HBeAg, antiHBe were tested by using commercially available enzyme linked immunosorbent assay kits (Organon Teknika, Boxtel, The Netherlands).

HBV DNA preparation and amplification

The sera were stored at -80°C until analysis. Viral DNA was extracted from 200 µL serum by phenol/Chloroform extraction after incubation with Proteinase K^[20]. HBV DNA was detected using in-house nested polymerase chain reactions (PCR) targeting the DNA sequences encoding the surface and the precore/core regions by the method described earlier^[16,17]. Instructions to prevent cross contamination were followed strictly^[21] and the results were considered valid only when they were consistently obtained in duplicate.

Genotyping and Sequencing of HBV

HBV genotyping was done in 230 HBV DNA isolates by Restriction Fragment Length Polymorphism (RFLP) using the methods described earlier^[5] and reconfirmed on the basis of phylogenetic analysis of the 440 nt fragment of S gene of 45 randomly chosen isolates. Of these 45 isolates, 27 were from genotype C, 9 from genotype A and 9 from genotype D. The nested PCR products were sequenced from both directions on an ABI Prism 377 (Applied Biosystems, Foster City, California, USA), using the PCR primers. BCP and precore core region were sequenced using primers as described earlier^[17].

Subgenotypes of HBV/C were assigned as described previously, C1 (Cs), C2 (Ce)^[7,8] and C3, C4^[10].

Phylogenetic analysis

For sequence alignment as well as phylogenetic analysis, we selected the GenBank sequences with the best and the high scoring matches with our sequences in a NCBI BLAST search. Sequences were edited, aligned and analyzed using Bioedit version 7.0.4.1^[22]. Genetic distances were calculated using the Kimura two parameter algorithm and phylogenetic trees were constructed by the neighbor joining (NJ) method. To confirm the reliability of the pair wise comparison and phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1000 times. Phylogenetic analysis was done using MEGA version 2.1^[23].

RESULTS

The clinical and demographic characteristics of 230 HBV carriers are shown in Table 1. Among them 200 patients (age between 5-68 years, 77 HBeAg+ and 123 HBeAg-) were native resident of Kolkata and its neighborhood, while 30 (age between 2-50 years, 3 HBeAg+ and 27 HBeAg-) were from rural areas. All the patients were ethnic Bengali. Median serum HBV DNA load of the patients was 5.37 (range 5.15-9.15) log copies/mL.

Table 1 Clinical and demographic characteristics of 230 HBV carriers from Eastern India

	HBV carriers from		Total
	Kolkata (n = 200)	Rural areas (n = 30)	
Age (yr)	32.56 ± 12.98	26.03 ± 14.14	31.71 ± 13.29
Sex (M/F)	173/27	21/9	194/36
Genotype (A/C/D)	57/40/103	0/2/28	57/42/131
ALT (IU/L)	83.24 ± 75.15	37.63 ± 21.73	77.29 ± 72.15
HBeAg positive	77	3	80
HBeAg negative	123	27	150

Table 2 Characteristics of 42 HBV/C isolates of Eastern India

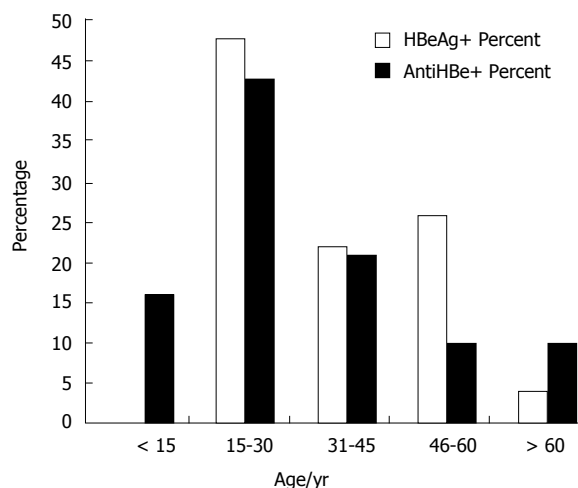
	HBeAg positive (n = 23)	HBeAg negative (n = 19)
Age (yr)	32.67 ± 13.04	32.47 ± 13.01
Sex (M/F)	19/4	15/4
ALT (IU/L)	75.13 ± 39.50	85.84 ± 54.14
Subtype adrq+/adw2	23/0	18/1
T1762/A1764	12 (52%)	17 (89%)
A1896	2 (9%)	4 (21%)

Genotype distribution

Three HBV genotypes (A, C and D) could be detected among the 230 HBV DNA positive samples studied by RFLP method. Majority of the samples belonged to Genotype D, 131/230 (57.0%), followed by genotype A, 57/230 (24.8%) and 42/230 (18.2%) isolates were identified as genotype C, the majority of which (40 of 42) were from outpatient clinics of Kolkata. Out of the 30 samples from incidentally detected asymptomatic carriers from rural areas, only 2 (2/30, 6.7%) were of genotype C, while the rest were of genotype D (93.3%) (Table 1). The banding pattern of all the genotype C samples was similar to that of genotype C pattern found among Southeast Asian carriers by Lind *et al* 1997.

Characteristics of 42 HBV/C isolated from Eastern India

Among the 42 HBV/C strain, 23 (53%) were HBeAg positive and rest 19 (47%) were antiHBe positive. In order to clarify the clinical characteristics of HBV/C carriers in this region clinical and laboratory data between HBeAg positive and antiHBe positive patients were compared (Table 2). There was no significant difference between the mean age of HBeAg positive and antiHBe positive group. The mean ALT level was slightly high in antiHBe positive (85.84 ± 54.14 *vs* 75.13 ± 39.50) cases but the difference was not statistically significant. T1762/A1764 double mutation in the BCP region was more frequent in antiHBe positive than HBeAg positive group (17/19, 89% *vs* 12/23, 52%) with elevated ALT level, whereas the frequency of A1896 mutation that creates a stop codon in the precore region was low 6/42 (14%). To examine the correlation between age and HBeAg/antiHBe status, the age specific prevalence of the HBeAg/antiHBe status in 42 HBV/C

**Figure 1** Age specific prevalence of HBeAg/antiHBe status in 42 HBV/C strain isolated from Eastern India.

subjects was analyzed (Figure 1). About 19/42 (45%) of subjects were in the age group of 16-30 years irrespective to HBeAg status. After that the infection rate gradually decreased.

Phylogenetic analysis

Phylogenetic analysis based on nucleotide (nt.) 256-696 of the Surface (S) gene region of HBV isolates was used to confirm the presence of these three genotypes from 45 isolates. The 45 S gene fragments were analyzed along with 39 reference sequences of different genotypes retrieved from GenBank in the phylogenetic tree presented in Figure 2. Genotypes A with adw2 subtype and D with ayw2 and ayw3 subtype from the present study clustered with the genotype A and D sequences previously reported from India. However, genotype C isolates with adrq+ and adw2 subtype from Eastern India clustered with HBV/Cs subgroup found in South East Asian countries rather than HBV/Ce subgroup found in the Far East like China, Japan, and Korea. Overall percent nucleotide identity (PNI) for HBV genotype C isolates from Eastern India with HBV/Cs varied from 98.4 to 100%. In addition to the above observation, 24 strains were most closely related to the Thai strain, AF068756 in the NCBI BLAST search. The PNI for these 24 sequences with AF068756 was found to be $99.5\% \pm 0.4\%$, across the 440 nt fragment in the S gene region. EI01343 and EI01329 were two HBV/C strain isolated from rural areas. Sequences have been submitted under GenBank accession numbers AY879184-AY879228.

Figure 3 represents the phylogenetic relatedness based on core sequences of the HBV/C strain from Eastern India. In this study, the core gene sequences were obtained from 19 HBV/C strain, 12 in this present study (accession no AY967430, AY967435, AY967442, AY967456-62, AY967465, AY967467) and 7 from previous study^[17]. The phylogenetic tree was also constructed using the core gene sequences (nt. 1900 to 2350) of 39 reference sequences of different genotypes retrieved from GenBank along with 19 HBV/C strain from Eastern India. It was evident that all

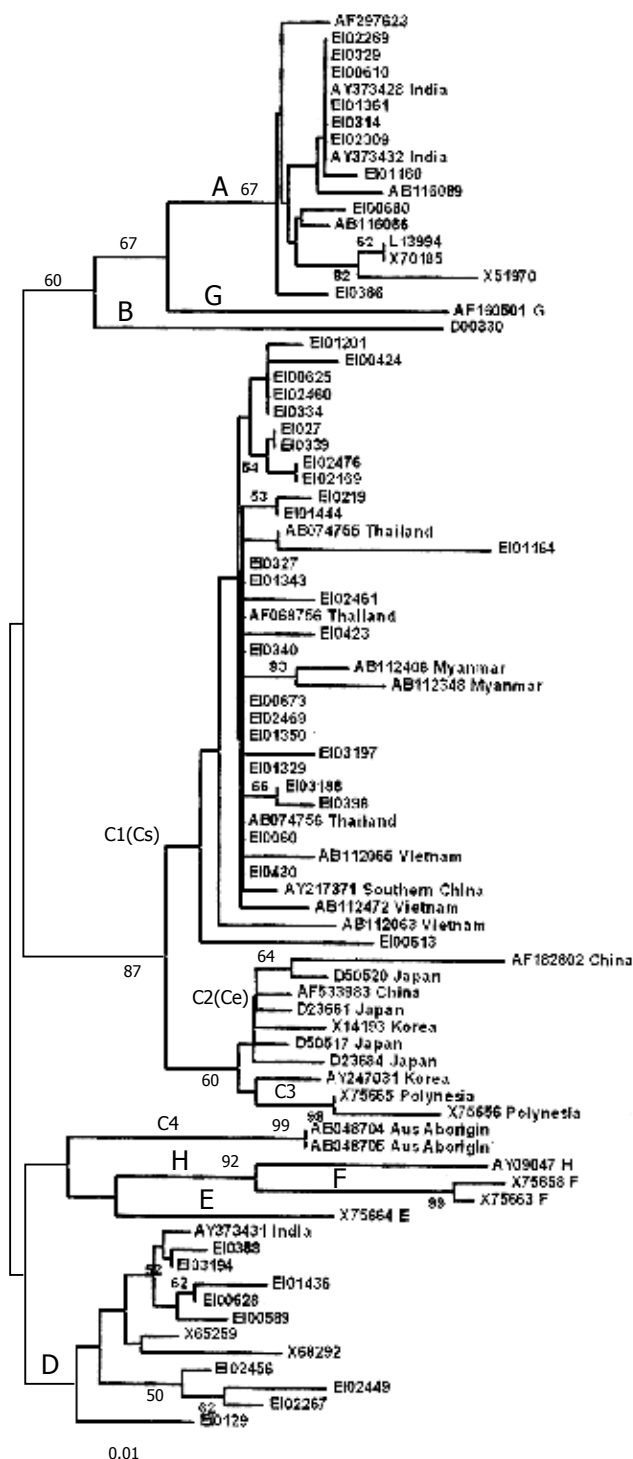


Figure 2 Neighbor joining phylogenetic of S gene region (codon 35-180) of HBV isolates from eastern India (denoted with EI) along with other genotypes derived from Genbank.

the HBV/C strain isolated from Eastern India clustered with South East Asian strain (HBV/Cs) and also found to be most closely related to Thailand sequences.

Mutation in S gene region

Amino acid sequences of the part of S protein (codon 41-180) of 27 HBV/C isolates from our study were compared with sequences retrieved from GenBank. Subtyping of the HBV isolates was done on the basis of presence of amino acid residues at codon 122, 127 and

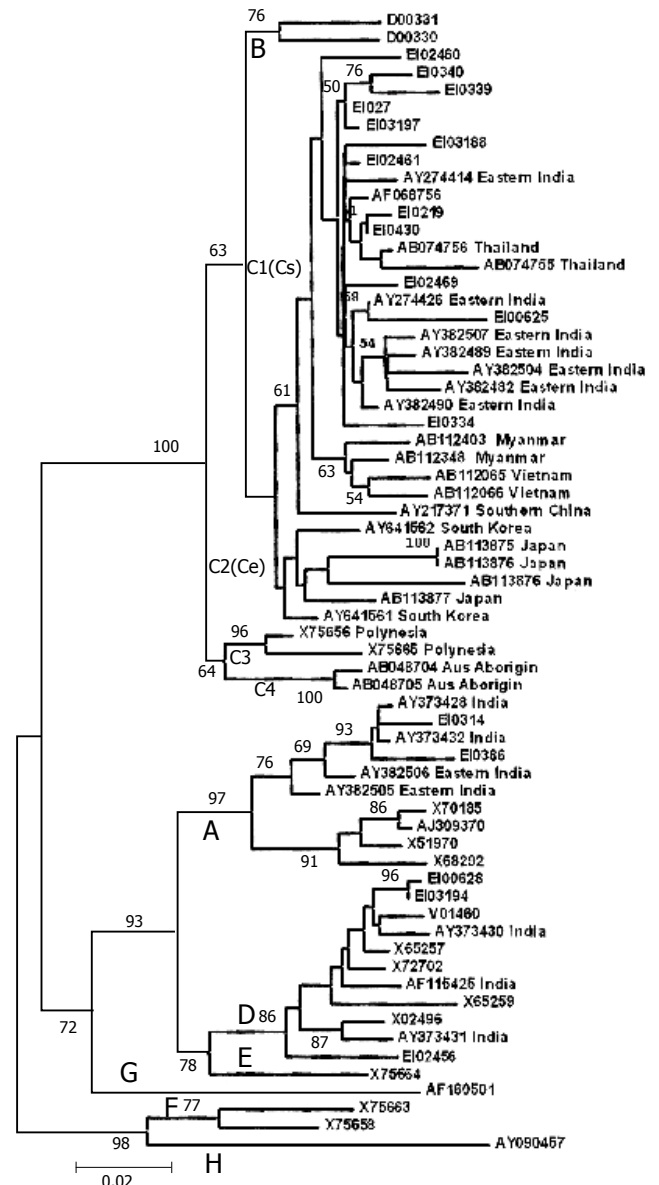


Figure 3 Neighbor joining phylogenetic analysis using the core gene region (nt. 1900-2300) of HBV isolates from Eastern India (denoted with EI).

160 of the S gene region (Figure 4A and B). The sub determinant q⁺ and q⁻ was differentiated on the basis of amino acid residue at codon 177 and 178. Among the genotype C isolates 26 out of 27 isolates had amino acid Lys122 and Arg160 as well as Val177 and Pro178 indicating that they were adr^{q+} subtype. However, only one isolate (EI01164) was classified as adr^{q-} subtype on the basis of amino acid Lys122 and Lys160 and Pro127 present in the antigenic determinant.

Comparison of amino acid sequences showed that most of the sequences were conserved (Figure 4A and B). Consensus amino acid substitution at codon 53 and 126 was observed within HBV/Cs and HBV/Ce. Out of 27 genotype C sample studied 20 were shown to have Ser to Leu amino acid substitution at codon 53, which was also found in all genotype HBV/Cs sequence from the database. The remaining 7 samples had Ser at 53 codon, which was the characteristic of genotype Ce. Most of the samples from Far East (HBV/Ce) had amino acid Ile at

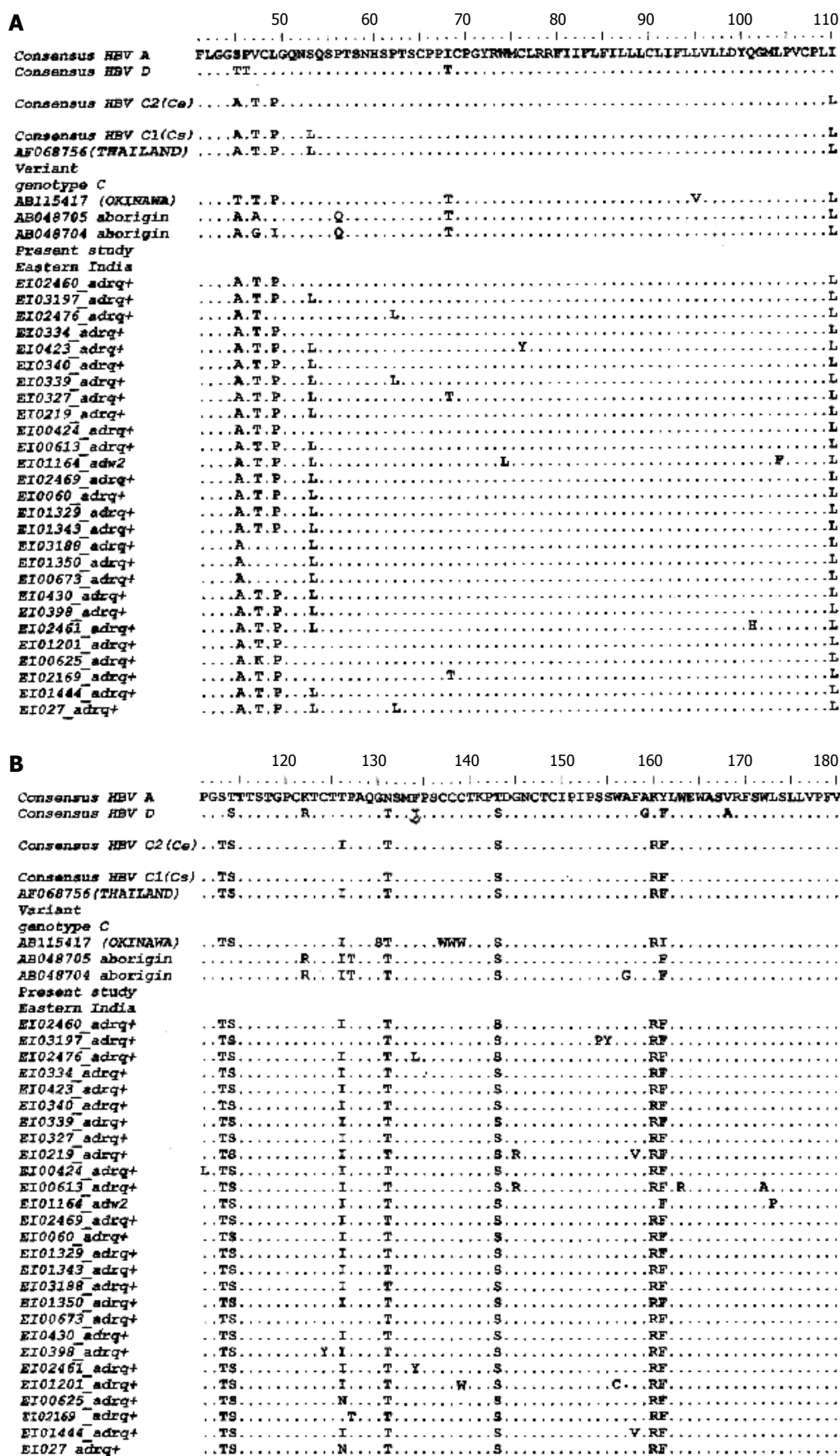


Figure 4 A, B: Alignment of amino acid sequences (41-180) of the partial S protein of HBV/C isolated from Eastern India.

codon 126, whereas most of the samples from Southeast Asia had either Asn or Thr at codon 126. However the isolate AF068756 from Thailand had Ile at codon 126. Major-

ity of the genotype C isolates studied by us also had Ile at codon 126. Only three isolates had Thr and two had Asn in that position.

DISCUSSION

Except for the HBV sequences from two studies that focused on Western and Northern India^[14,15], there are hardly any HBV sequences available from our vast country. In this study, HBV DNA sequencing and phylogenetic analysis of S gene and core region established the presence of genotype C among the HBV carriers in addition to HBV/A and HBV/D from Eastern India. This is in contrast to Western and Northern India where genotypes D and A are prevalent^[14,15]. Moreover, genotype C could be detected both from Kolkata as well as from the incidentally detected asymptomatic carriers from the rural population. Vivekanandan *et al* 2004 reported the presence of genotype C (by RFLP) only among chronic patients who went to their hospital for treatment from Eastern India. However, they could not detect HBV/C among their asymptomatic relatives who were incidentally detected HBsAg positive during blood donation. On the other hand, HBV genotypes found among patients who came from Southern India to that hospital were of genotype D excepting one case. However they have not characterized the molecular features of the genotype C strains of Eastern India. Taking into consideration their report, our findings, as well as other reports from Western and Northern India, it seemed likely that genotype C is at present confined mostly to Eastern India. Similar significant difference in the geographic distribution of HBV genotypes was recently reported from Japan and USA^[24,25].

Four subtypes adrq-, ayr, adw, adrq+ were associated with genotype C. Recently, association of serotype ayw3 was also found with genotype C variant, among Australian aborigines^[9]. In our study, in addition to adrq+ subtype, one (EI01164) of the genotype C HBV isolates showed adw2 subtype. High prevalence of this adw2 subtype within genotype C was already reported from Tibet, East Asia^[26] which borders Northern India. Genotypes A and D in our study group were of adw2 and ayw2, ayw3 subtype respectively which is consistent with the previous study reported from other parts of India^[14,15].

In places where well-known waves of migration have occurred over time, prevalence of HBV genotypes is known to reflect anthropological history of human migration, origin of immigrants and other patterns of migration. Thus, an apparent south-to-north gradient of genotypes C and B in Japan is considered to reflect anthropological history of migration from Asian countries to Japan^[24]. In contrast, the presence of genotype D HBV among primitive tribes of Andaman and Nicobar islands is thought to be due to introduction of HBV from mainland India in the past century^[27]. However, carrying the HBV/Cs strain among Jarwas (an isolated tribe from Andaman and Nicobar islands) correlate with the anthropological history of migration of this tribes from Southeast Asia^[28].

Population groups of Northern, Western and Eastern India ethnically are of Caucasoid origin, who speak Indo-European languages and show close genetic affinities with populations of Eurasia and Europe^[29] where HBV genotypes A and D are prevalent. It is therefore not surprising that HBV genotype D and A is most predominant in India. On the other hand genotype C is predominant in countries

neighboring Eastern part of India, where the population groups (of Mongoloid origin) are believed to have originated from Tibeto-Burman language subfamily. Genetic study showed that the Bengali population group of Eastern India had close ethnic affiliation with Caucasoids^[30] and formed clusters distinctly different from the North East population groups (ethnic affiliation to Mongoloid) who are possibly descendants of ancestral population of China, where genotype C and B are prevalent. Since our study population is not of Mongoloid origin, therefore it is quite unlikely that presence of genotype C in Eastern India reflects the history of population migration long ago.

This is further supported from the fact that when the sequences from the present study were compared to GenBank sequences in a BLAST search, the best matches and the high-scoring matches were from Southeast Asian Countries, especially from Thailand and not from China. Phylogenetic analysis both from Surface and core gene also revealed that almost all sequences from our study group clustered with Southeast Asian HBV/Cs subgroup and not with HBV/Ce found in East Asia and predominant in China. Of the 27 genotype C isolates sequenced, 24 (90%) had percent nucleotide identity (PNI) of 99.5% \pm 0.4%, with Thai genotype C sequence (Accession No. AF068756), in the S gene region.

There are several reports available where low genetic diversity of HBV strains has been considered to suggest limited and relatively recent spread of the strain over the geographical region. Arankelle *et al*^[27] reported a recent introduction of the virus, on the basis of low genetic diversity of partial S gene (PNI varied from 1.6% to 2.0%), of genotype D isolates from mainland India to tribal population of Andaman and Nicobar islands. For similar reason, HBV/E was considered as relatively recent introduction in West Africa^[31]. Thus considering low genetic diversity of HBV/C strain in Eastern India with Thai sequences, we presumed that the genotype Cs in Eastern India possibly might have spread from Southeast Asia, particularly from Thailand, rather recently. It is noteworthy that the prevalence of genotype C is much higher in urban population (20% *vs* 6.7%) than in rural population. Moreover, genotype C HBV samples have not been reported from Northern and Western parts of India^[14,15]. In the Southern Indian Tertiary hospital^[18], genotype C was detected in a significantly higher proportion of patients from Eastern India compared with those from Southern India (16.8% *vs* 0.9%, $P < 0.0001$). This selective confinement of genotype C to Eastern India especially to the urban population suggests that perhaps sufficient time has not yet passed since its introduction to Eastern India for its subsequent spread to the rest of the country. However, we must admit that this introduction of genotype C in Eastern India would have been best addressed if sequential study showing changing HBV genotype pattern could have been documented in Eastern India.

Kolkata is the most important port city of Eastern India and proximal to Southeast Asia. Increased trade relationship with Thailand suggests that spread of genotype Cs to Eastern India is possible, but the infectious source and the route of transmission are not clear. Previous reports showed presence of Thai HIV strains as well as Thai

HCV strains^[32,33] from North Eastern and Eastern India. Heroin trafficking routes have been associated with IDU and HIV infection in Thailand, Myanmar, China and India^[34]. HBV and HIV share common modes of transmission. There is possibility of co transmission of HBV from Southeast Asia to Eastern India, *via* this route. This might have been followed by spread to general population via unsafe injection practices, which is common in developing countries including India^[35,36]. The presence of HBV/Cs is known in our neighboring country Bangladesh, however the proportion of HBV/C among HBV carriers in Bangladesh, is unknown^[10]. People from Bangladesh come to Kolkata for treatment. This population might also have some influence in the presence of genotype C in Kolkata. Whatever may be the source, this study confirmed the presence of Cs in the Eastern Indian population.

In an era of frequent international travel and human migration, introduction of new HBV genotype to a community might have far reaching effects, including recombination between genotypes^[37] or replacement of one genotype by another^[38]. HBV genotype C is associated with delayed hepatitis B e antigen (HBeAg) seroconversion^[39], more-active hepatitis^[40], lower response to antiviral therapy^[41], more advanced liver disease and a higher risk of hepatocellular carcinoma^[42], compared with HBV genotype B. Furthermore, HBV/C with T1762/A1764 mutation in BCP region has been reported to be an increased risk factor for hepatocellular carcinoma^[13]. In our study, at least 45% of the subjects were in the age group of 16-30 years. In addition, 29/42 (69%) of genotype C isolates had T1762/A1764 mutation in BCP region, most of them associated with elevated ALT. Thus, the patients infected with genotype C need to be carefully monitored to assess their clinical outcome in future.

In conclusion, the present study reports the genotypic distribution and partial genome sequences of HBV/C isolates from Eastern India in addition to most predominant HBV/A and HBV/D. These isolates clustering with HBV/Cs genotype found in South East Asia, is prevalent in considerable proportion (18%) of Eastern Indian HBV carriers. Low genetic diversity and confinement of HBV/C in Eastern India possibly indicates a recent, limited, spread of HBV/Cs in this region. Several studies have suggested that HBV genotype C is associated with more active or severe sequelae of liver disease in Southeast Asia compared with genotype B. Therefore, the presence of HBV genotype C in Eastern India should be carefully monitored by further studies including epidemiological, clinical and virological assessment.

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H pylori

***H pylori* stimulates proliferation of gastric cancer cells through activating mitogen-activated protein kinase cascade**

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Abstract

AIM: To explore the mechanism by which *H pylori* causes activation of gastric epithelial cells.

METHODS: A VacA (+) and CagA (+) standard *H pylori* line NCTC 11637 and a human gastric adenocarcinoma derived gastric epithelial cell line BGC-823 were applied in the study. MTT assay and ³H-TdR incorporation test were used to detect the proliferation of BGC-823 cells and Western blotting was used to detect the activity and existence of related proteins.

RESULTS: Incubation with *H pylori* extract increased the proliferation of gastric epithelial cells, reflected by both live cell number and DNA synthesis rate. The activity of extracellular signal-regulated protein kinase (ERK) signal transduction cascade increased within 20 min after incubation with *H pylori* extract and appeared to be a sustained event. MAPK/ERK kinase (MEK) inhibitor PD98059 abolished the action of *H pylori* extract on both ERK activity and cell proliferation. Incubation with *H pylori* extract increased c-Fos expression and SRE-dependent gene expression. *H pylori* extract caused phosphorylation of several proteins including a protein with molecular size of 97.4 kDa and tyrosine kinase inhibitor genistein inhibited the activation of ERK and the proliferation of cells caused by *H pylori* extract.

CONCLUSION: Biologically active elements in *H pylori* extract cause proliferation of gastric epithelial cells through activating tyrosine kinase and ERK signal transduction cascade.

INTRODUCTION

H pylori is an important pathogen associated with gastritis and peptic ulcers^[1]. It has also been defined as a carcinogen^[2]. The mechanisms of pathogenic and carcinogenic effects of *H pylori* infection are under intensive investigation. Research data suggest that *H pylori* might stimulate the proliferation of gastric epithelial cells both *in vitro* and *in vivo*^[3-5]. This effect of *H pylori* has an important pathogenic significance because increased cell proliferation may elevate the gastric mutation rate and is a predisposing factor for neoplasia. The mechanism, especially the intracellular signal transduction pathway associated with the stimulating effect of *H pylori* on the proliferation of gastric epithelial cells, is under extensive study.

Protein kinases, which regulate the protein phosphorylation, are considered to play the most important role in regulating protein function and cell activity. Mitogen-activated protein kinase (also known as MAP Kinase) is a crucial member of the protein kinase family. So far, four MAP Kinase cascades have been characterized in mammalian cells. Among them, extracellular signal-regulated protein kinase (ERK) cascade is most well characterized. The activation of signal transduction pathways by growth factors, hormones and neurotransmitters is mediated through ERK cascade^[6,7]. It is the basic signal transduction pathway regulating cell proliferation and differentiation. Recent research data indicate that activation of ERK cascade is involved in *H pylori*-induced proliferation of gastric epithelial cells^[8-10]. However, more details of *H pylori*-induced activation of ERK cascade are needed to be explored.

MATERIALS AND METHODS

Materials

Human gastric epithelial cell line BGC-823 (ICLC

HTL98007) was provided by Institute of Tumor Research of Beijing. VacA (+) and CagA (+) international standard *H. pylori* strain NCTC11637 was provided by Institute of Microbiology and Epidemiology of China, Beijing. DMEM culture medium was from Gibco (Grand Island, NY). Brucella broth was from Becton Dickinson (Franklin Lakes, NJ). New born calf serum (NBCS) was from No.2 Factory of Beijing Milk Company (Beijing, China). Antibody against phosphorylated ERK was from Sigma (St. Louis, MO). All other primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated secondary antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA). ECL chemoluminescence reagents were from Amersham (Buckinghamshire, England). Tyrosine kinase inhibitors genistein and luciferin were from Sigma (St. Louis, MO). MAPK/ERK inhibitor PD98059 was from Calbiochem (San Diego, CA). Plasmid DNA constructs encoding SRE-luciferase and CRE-luciferase were kind gifts from Dr. Renate Pilz in University of California, San Diego.

Cell culture

Human gastric cancer cell line BGC-823 was kept in DMEM containing 100 mL/L serum and incubated in 5% CO₂ at 37°C. The medium was changed every second day and the cells were sub-cultured at 80%-90% confluence.

Preparation of *H. pylori* extract

Healthy *H. pylori* was scraped down from horse serum agar plate and transferred into Brucella broth containing 100 mL/L serum. The bacterial suspension was incubated in micro-oxygen environment (80% N₂, 15% CO₂, 5% O₂) at 37°C for 24 h with continuous shaking. The growth of *H. pylori* was confirmed by Gram staining before harvest. *H. pylori* cells were precipitated by centrifugation at 5000 r/min for 15 min at 4°C and washed with PBS. The bacteria were re-suspended in serum free cell culture medium (DMEM) and sonicated (100 W, 15 s × 6, at 30 s interval). The breakdown of the bacteria was confirmed by microscopy. The suspension was centrifuged at 10000 r/min for 30 min at 4°C. The supernatant (*H. pylori* extract) was adjusted to protein concentration of 1 g/L and kept at -20°C until use.

Preparation of protein samples

The harvested cells (1×10^7) were washed three times with PBS and re-suspended in 40 µL suspension buffer containing 100 mmol/L NaCl, 10 mmol/L Tris-HCl (pH7.6), 1 mmol/L EDTA (pH8.0), 1 mg/L aprotinin, 100 mg/L PMSF. The same volume of boiled 2 × SDS protein loading buffer containing 100 mmol/L Tris-HCl (pH6.8), 200 mmol/L DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol was added into the cell suspension. The sample was boiled for 10 min to lyse the cells and sonicated for 1 min to break down the DNA. After centrifugation at 12000 r/min for 10 min, the supernatant was kept at -20°C until use. For preparation of membrane protein samples, the cells were harvested, washed with PBS, and lysed with extract buffer containing 20 mmol/L HEPES (pH7.4), 10

mL/L Triton X-100, 5 mmol/L EDTA, 50 mmol/L NaCl, 30 µmol/L β-glycerophosphate, 50 mmol/L sodium fluoride, 50 mg/L aprotinin, 10 mg/L leupeptin. The lysate was centrifuged at $100000 \times g$ for 1 h at 4°C and the supernatant was kept at -20°C until use.

Western blotting

The protein sample was loaded with 30 µg protein per lane on SDS-PAGE gel. After electrophoresis, the proteins on the gel were transferred onto nitrocellulose (NC) membrane as described previously. The NC membrane was blocked with 50 g/L milk in TBS-T for 1 h at room temperature (RT), incubated with first antibody for 2 h and with secondary antibody for 50 min at RT and finally washed three times after each incubation. ECL chemoluminescence reagent was used to show the positive bands on the membrane.

MTT assay

A total of 1×10^4 trypsin-dispersed BGC-823 cells in 0.1 mL culture medium were seeded into each well of the 96-well plates and cultured for 24 h. Then, the cells were incubated with medium alone or with medium plus *H. pylori* extract at different concentrations. After 24 h of further incubation, 10 µL of MTT (6 g/L, Sigma) was added to each well and the incubation was continued for 4 h at 37°C. Finally, the culture medium was removed and 200 µL of dimethylsulphoxide (DMSO) was added to each well. The absorbance was determined with an ELISA reader at 570 nm. The “change percentage” of the absorbency (*A*) value of the cells treated with *H. pylori* extract compared with *A* value of the cells without treatment was calculated as:

$$\text{Change (\%)} = \frac{A \text{ value (} H. pylori \text{ group-control)}}{A \text{ value of control group}} \times 100\%$$

³H-TdR incorporation

The cells were cultured in the same way as described in MTT assay. When the culture medium was changed and *H. pylori* extract was added, 0.2 µCi of ³H-TdR was also added into each well. After further incubation for 24 h, the medium was discarded and the cells were trypsin-dispersed and collected onto a membrane. The membrane was heated dry at 65°C and put into a plastic vial containing 6 mL of scintillation liquid. The count per minute (CPM) was calculated with a Beckman scintillation counter. The “change percentage” of CPM of the cells treated with *H. pylori* extract compared with CPM of the cells not treated with *H. pylori* extract was calculated as:

$$\text{Change (\%)} = \frac{\text{CPM (} H. pylori \text{ group-control)}}{\text{CPM of control group}} \times 100\%$$

Reporter gene assay

The cells were transfected with plasmid DNA encoding SRE-luciferase or CRE-luciferase, and β-galactosidase. After incubation with *H. pylori* extract for 8 h, the cells were harvested and the activity of luciferase and β-galactosidase was measured by luminescence-based assays. The luciferase activity was calculated as the fold of increase after normalized by the β-galactosidase activity.

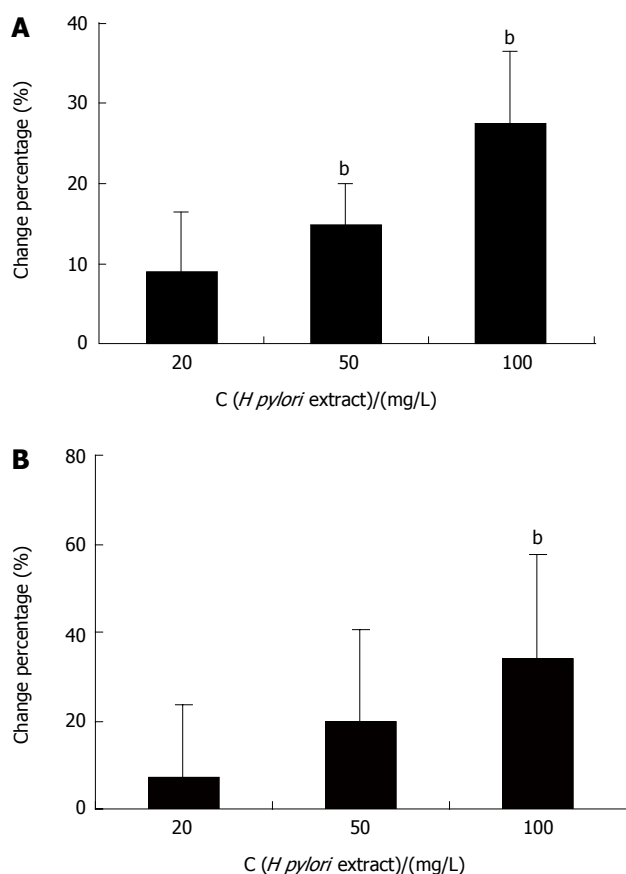


Figure 1 MTT assay (A) and ³H-TdR-incorporation test (B) showing *H pylori* extract-stimulated proliferation of BGC-823 cells. ^b*P* < 0.01 vs control.

Statistical analysis

The results of MTT assay, ³H-TdR incorporation test and reporter gene assay data were expressed as means ± SD. The significance of the difference between control group and experimental group was evaluated by Student's *t*-test.

RESULTS

H pylori extract stimulated proliferation of BGC-823 cells

The MTT assay showed that *H pylori* extract could stimulate the proliferation of BGC-823 cells. The *A* value reflecting the number of viable cells, increased significantly in the cells incubated with 50 mg/L and 100 mg/L *H pylori* extract for 24 h (Figure 1A). Similar result was also obtained in ³H-TdR-incorporation test. The ³H incorporation increased dose-dependently when the cells were incubated with 20 mg/L, 50 mg/L, and 100 mg/L *H pylori* extract respectively for 24 h (Figure 1B).

H pylori extract activated extracellular signal-regulated kinase in BGC-823 cells

The separated protein samples from cells incubated with 50 mg/L *H pylori* extract for different periods of time were first probed with antibody against phosphorylated ERK to analyze the activity of ERK and re-probed with anti-ERK2 antibody to show the protein content of this kinase. The results showed that in serum-starved cells, the activity level of ERK was very low. During incubation with *H pylori* extract, the activity of ERK increased obviously

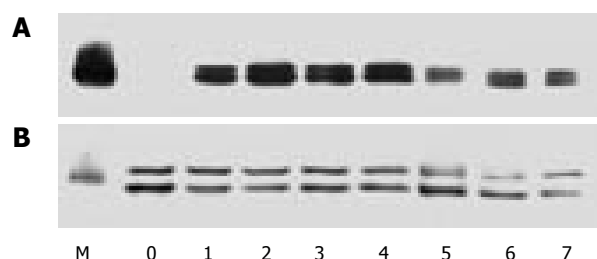


Figure 2 Western blotting showing phosphorylated ERK (A) and total ERK (B) in serum-starved BGC-823 cells incubated with *H pylori* extract. M: Protein molecular marker; 0: Control; 1-7: Incubation with 50 mg/L *H pylori* extract for 20, 40, 60 min and 3, 6, 12, 24 h.

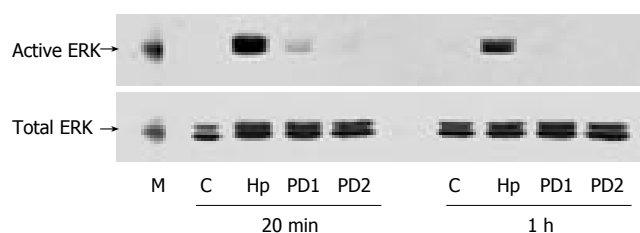


Figure 3 Western blotting showing PD98059-blocked stimulating effect of *H pylori* extract on ERK activation in BGC-823 cells. M: Molecular marker; C: Control; Hp: 50 mg/L *H pylori* extract; PD1: 25 μmol/L PD98059 + 50 mg/L *H pylori* extract; PD2: 50 μmol/L PD98059 + 50 mg/L *H pylori* extract.

within 20 min and stayed at a high level (about 5 times of control) for more than 8 h (Figure 2).

PD98059 blocked the stimulating effect of H pylori extract on ERK activity and proliferation of BGC-823 cells

PD98059, an inhibitor of MAPK/ERK kinase (MEK) activating ERK directly, was used to confirm the specificity of ERK activation induced by *H pylori* extract. Western blotting showed that PD98059 abolished the stimulating effect of *H pylori* extract on ERK activity (Figure 3). MTT assay showed that the inhibitor also blocked the stimulating effect of *H pylori* extract on proliferation of the cells (Figure 4).

Genistein inhibited ERK activation induced by H pylori extract

Genistein is an inhibitor of tyrosine kinase. Western blotting with antibody against phosphorylated ERK showed that treating the cells with genistein inhibited the ERK activation induced by *H pylori* extract (Figure 5).

H pylori extract caused tyrosine phosphorylation of membrane protein

An antibody against pan-tyrosine phosphorylation of proteins was used to detect the tyrosine phosphorylation caused by *H pylori* extract. Western blotting showed that during incubation with *H pylori* extract, several proteins were phosphorylated on tyrosine within 20 min of incubation (Figure 6). A tyrosine-phosphorylated protein with molecular size around 97.4 kDa was especially arrested because it also existed in membrane protein sample extracted from the cells (Data not shown).

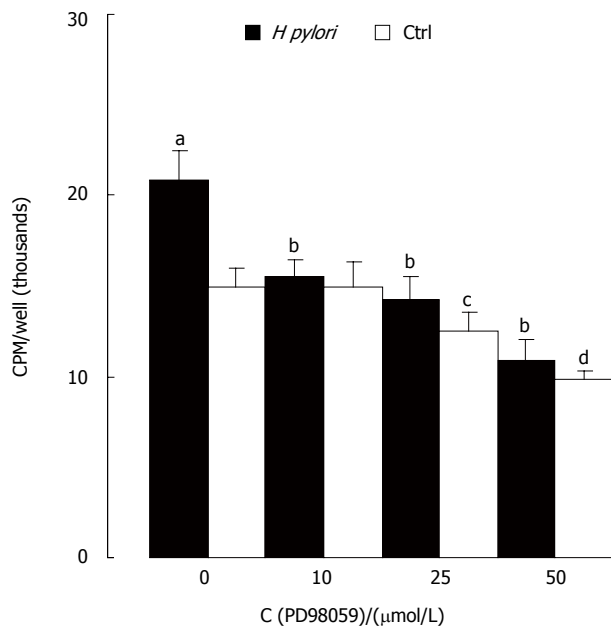


Figure 4 MTT assay showing PD98059-blocked proliferation-stimulating effect of *H pylori* extract. ^a*P* < 0.05, ^c*P* < 0.05, ^b*P* < 0.01 vs control only; ^d*P* < 0.01 vs Hp only.

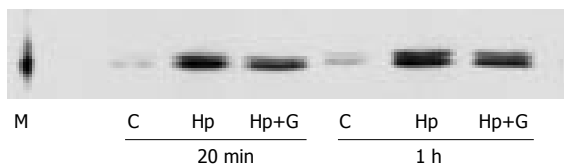


Figure 5 Western blotting showing genistein-prevented ERK activation by *H pylori* extract. M: Molecular marker; C: Control; Hp: *H pylori* extract; Hp + G: Genistein and *H pylori* extract.

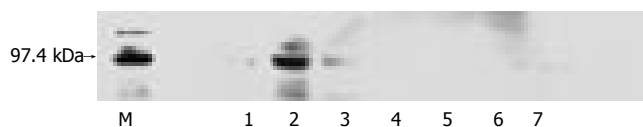


Figure 6 Western blotting showing *H pylori* extract-caused tyrosine phosphorylation of cell lysate of BGC-823. M: Molecular marker; 1: control; 2-7: *H pylori* extract incubated for 20, 40 min and 1, 3, 6, 12 h.

H pylori extract increased expression of c-fos

Western blotting showed that when the cells were incubated with 50 mg/L *H pylori* extract, the expression of c-Fos protein increased obviously. During incubation, the expression of c-Fos started to increase around 40 min after incubation, reached its peak around 1 h and lasted for 6 h (Figure 7).

H pylori extract stimulated SRE-dependent reporter gene expression

Reporter gene assay showed that *H pylori* extract specifically stimulated SRE-dependent expression of luciferase while it had no effect on CRE-driven luciferase expression (Figure 8), indicating that *H pylori* extract could increase c-Fos expression through SRE cis-element of the promoter.

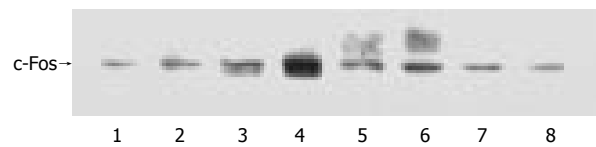


Figure 7 Western blotting showing *H pylori* extract-increased expression of c-Fos. 1: Control; 2-8: *H pylori* extract incubated for 20, 40 min and 1, 3, 6, 12 and 24 h.

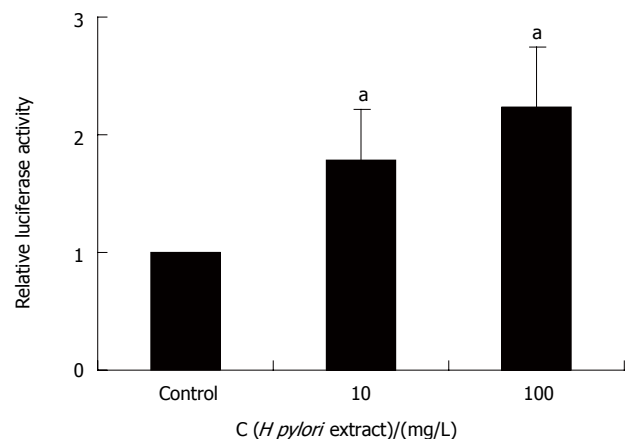


Figure 8 *H pylori* extract-stimulated SRE-dependent reporter gene expression. ^a*P* < 0.05 vs control.

DISCUSSION

Gastric *H pylori* infection is the most common infection in humans. It is responsible for virtually all cases of gastritis and most cases of peptic ulcers^[1]. Moreover, *H pylori* is defined as a group 1 carcinogen in humans by International Agency for Research on Cancer, a working party of WHO^[2]. The conclusion is, however, mainly based on the epidemiological data and clinical investigations. Even though laboratory data on carcinogenesis of *H pylori* are accumulating during recent years^[11-13], more efforts are still needed to elucidate the mechanism of *H pylori*-induced malignant diseases. Gastric carcinoma develops through a sequence of events from normal mucosa to gastric carcinoma^[14]. *H pylori* might be closely associated with this process. Several mechanisms have been proposed by which *H pylori* infection might lead to predisposition for gastric cancer^[15]. One explanation is that *H pylori*-associated chronic inflammation may increase the rate of cell turnover in gastric mucosa. Rapidly replicating DNA is more susceptible to damage. Alongside increased reactive oxygen species and decreased ascorbic acid content during inflammatory reactions, *H pylori*-induced proliferation may be the major factor for gastric dysplasia and gastric cancer^[16,17]. In *H pylori* hosts with gastritis, increased proliferation of gastric epithelial cells has been confirmed^[3,4]. Laboratory data have also shown that *H pylori* might directly stimulate proliferation of gastric epithelial cells^[5,10,11]. The former reveals an increased cell turnover rate in *H pylori* host and the latter suggests that increased proliferation of gastric epithelial cells might be a direct effect of *H pylori*.

Probing separated proteins with anti-phosphorylated ERK antibody and re-probing them with anti-ERK2

antibody provide a sensitive way to detect ERK activity^[18]. With this method, our study showed that during incubation with *H pylori* extract, the activity of ERK increased obviously in serum-starved cells and the activation of ERK was a sustained event. This is significant because sustained activation of ERK appears to be required for many cells to pass the gastrointestinal restriction point to enter S phase, in which cellular DNA is replicated^[19,20]. MTT assay and ³H-TdR-incorporation test confirmed the stimulating effect of *H pylori* extract on cell proliferation in our study. Since ERK-mediated cascade is the basic signal transduction pathway regulating cell proliferation, this pathway can mediate proliferation-stimulating effects of *H pylori*.

ERK is in the middle of the signal transduction pathway. Currently, it is believed that ERK may be activated through several pathways^[21]. One is the receptor tyrosine kinase-Ras-MEK pathway. Growth factor ligand binds to membrane receptor and causes tyrosine-phosphorylation of the receptor. Consequential action of Ras and then Raf, which are components of the cascade, causes the activation of MEK (also known as MAP kinase), a direct upstream regulator of ERK^[22]. PD98059 is a specific inhibitor of MEK^[23]. Our results showed that PD98059 could block both ERK activity-stimulating and proliferation-stimulating effects of *H pylori* extract. Ras activity was not investigated in this experiment. However, we used tyrosine kinase inhibitor genistein to explore the possible involvement of tyrosine kinase in *H pylori* extract-induced signaling. The results showed that genistein inhibited *H pylori* extract-induced activation of ERK. Then, we used antibody against pan-tyrosine phosphorylation to detect tyrosine phosphorylation in both whole cell lysate and membrane protein from the cells. The results showed that in samples from the cells treated with *H pylori* extract, there was obvious tyrosine phosphorylation of a membrane protein with molecular size around 97.4 kDa, suggesting that it is very likely that *H pylori* extract activates ERK through receptor tyrosine kinase-Ras-MEK pathway.

As to the down-stream events of ERK activation, it was reported that some ERK activated by MEK can translocate to the nuclei and phosphorylate at least two transcription factors, c-Myc and Elk-1^[24,25]. The phosphorylated factors may lead to increased production of c-Fos mRNA and finally lead to cell activation^[26]. Mitsuno *et al*^[27] reported that co-culturing human gastric cancer cells with Cag-positive *H pylori* strains can activate c-fos gene through SRE. Our results showed that *H pylori* extract stimulated SRE-dependent gene transcription and increased the expression of c-Fos protein, indicating that the signaling process behind activation of ERK by *H pylori* can derive biological elements.

Several methods, including co-culture of live *H pylori* with target cells, use of water extract and sonication extract to stimulate target cells, have been used to study the effect of biologically active components of *H pylori* on the activity of target cells. For example, during co-culture, *H pylori* may secrete active factors or act on the target cells through type IV secretion system^[28], water extracting can get the surface component of the bacteria^[29], and sonication extracting can efficiently release

active components from the body of bacterial cells^[30,31]. In this experiment, sonication of *H pylori* extract showed that soluble component from the bacteria activated the MAPK cascade of gastric cells. Further work is needed to identify the molecular features of the component in our laboratory.

In summary, *H pylori* extract has direct proliferation-stimulating effects on gastric epithelial cells and the activation of gastric epithelial cells can be induced by *H pylori* through MAPK-mediated signal transduction pathway, suggesting that biologically active elements in *H pylori* contribute to the tumorigenesis effect of the bacteria.

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BASIC RESEARCH

***Lactobacilli*, *bifidobacteria* and *E. coli* nissle induce pro- and anti-inflammatory cytokines in peripheral blood mononuclear cells**

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Abstract

AIM: To investigate whether the stimulation of peripheral blood mononuclear cells (PBMNC) with the cell debris and cell extraction of different probiotic strains is similar or species specific.

METHODS: Three strains of *bifidobacteria*, 4 strains of *lactobacilli*, and *E. coli* nissle were sonicated and centrifuged in order to divide them into cell extract and cell debris. PBMNC were separated by density gradient and incubated for 36 h with either the cell debris or the cell extract of single strains of probiotic bacteria in doses from 10^2 to 10^8 CFU/mL. Cell supernatants were taken and interleukin (IL)-10, IL-1 β , and tumor necrosis factor (TNF)- α were determined by ELISA.

RESULTS: Depending on the species super-family, the strains had different stimulation patterns. Except for both *L. casei* strains, the cell extract of *bifidobacteria*

and *lactobacilli* had less stimulating capacity than cell debris, whereas the cell extract of *E. coli* nissle had similar stimulating properties to that of the cell debris of the strain and significantly more stimulating capacity than that of *bifidobacteria* and *lactobacilli*. The cell debris of *bifidobacteria* stimulated more cytokine release than the cell debris of *lactobacilli*. The cell debris of *lactobacilli* did not have a stimulating capacity when lower concentrations were used. Neither cell extraction nor cell debris had an inhibitory effect on the production of the tested cytokines by stimulated PBMNC.

CONCLUSION: The incubation of probiotic strains, which have been used in clinical trials for inflammatory diseases, with immunocompetent cells leads to different species specific reactions. High IL-10 response to cell debris of *bifidobacteria* and *E. coli* nissle can be found. This corresponds to positive effects of *bifidobacteria* and *E. coli* nissle in clinical trials for inflammatory bowel disease compared to negative outcomes obtained with *lactobacilli*.

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Key words: *Lactobacilli*; *Bifidobacteria*; Probiotics; Interleukin-10; Tumor necrosis factor- α ; Interleukin-1 β ; Peripheral blood mononuclear cells

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INTRODUCTION

There is no doubt that the relationship between intestinal microflora and immune system is complex. The host has to distinguish between pathogenic bacteria and harmless commensal and must react in an adequate and not self-destructive manner^[1]. There is an increasing body of evidence that chronic intestinal inflammations such as

inflammatory bowel disease (IBD) are due to a disturbed relationship within the host's immune response to the enteric microflora^[2-4]. Based on these proposals the relationship between micro-flora and intestinal immune response has been intensively studied by manipulation of the enteric micro-flora with probiotic bacteria^[5-11]. These are by definition "A preparation of or a product containing viable, defined products in sufficient numbers which alter the microflora (by implantation or colonization) in a compartment of the host and by exerting beneficial health effects in the host"^[12]. The efficacy of *E. coli* nissle in therapy for ulcerative colitis^[5-6] has been shown and our own clinical experience has been focused on a highly concentrated probiotic preparation (VSL#3) in preventing pouchitis, an unspecific inflammation of an ileal pouch anal anastomosis after colectomy for ulcerative colitis^[9-10]. *Lactobacillus* GG instead has no influence on clinical outcome in Crohn's disease^[7-8]. Besides clinical findings, different studies have also shown the influence of probiotic bacteria on the local and systemic immune response in experimental colitis^[13-15]. The mechanisms underlying this effect are still under investigation. One hypothesis is based on modulating pro- and anti-inflammatory cytokines^[16-17]. Pro-inflammatory cytokines such as interleukin (IL)-1 β and tumor necrosis factor (TNF)- α play an important role in gut inflammation^[17]. In Crohn's disease and ulcerative colitis these cytokines are elevated in local inflammation area and peripheral blood cells^[18-19], whereas the anti-inflammatory cytokine IL-10 is decreased in patients suffering from IBD^[20]. IL-10 produced by gene manipulated bacteria, reduces toxic colitis that is associated with pro-inflammatory cytokines^[21-22]. Further arguments have been found in the absence of IL-10. IL-10 deficient (KO) mice do not show any symptoms of intestinal inflammation as long as they are kept in sterile conditions, but spontaneously develop chronic colitis with a histological distribution similar to that found in Crohn's disease after termination of the sterile conditions^[23]. If the mice are fed with different lactic acid bacteria before finishing the sterile conditions, this chronic inflammation can be prevented^[24]. Recently, during preventive therapy for chronic pouchitis using different probiotics as mentioned above, we investigated the cytokine tissue levels of patients with pouchitis and after induction of remission and during the following probiotic application. It is interesting to find that cytokine tissue levels of IL-10 increase during the application of the probiotic preparation, whereas anti-inflammatory cytokine-levels such as TNF- α and IL-1 remain low after the application^[25].

The aim of our study was to set up an *in vitro* model to compare the immunomodulatory effects of different probiotic strains that have previously been evaluated in different clinical trials. For this purpose we used peripheral blood mononuclear cells (PBMNC), which are a combination of different immunogenic cells.

MATERIALS AND METHODS

Subjects

Blood samples were taken from 12 healthy blood

Table 1 List of the bacterial strains

<i>Bifidobacterium breve</i> : Y 8
<i>Bifidobacterium infantis</i> : Y 1
<i>Bifidobacterium longum</i> : Y 10
<i>E. coli</i> : Stamm Nissle 1917 (Mutaflor, Ardeypharm, Herdecke, Germany)
<i>Lactobacillus azidophilus</i> : MB 443
<i>Lactobacillus casei</i> subspecies <i>rhamnosus</i> : Lactobacillus GG (LGG, Valio, Helsinki, Finland): ATCC 53103
<i>Lactobacillus casei</i> : MB 451
<i>Lactobacillus delbrueckii</i> subspecies <i>bulgaricus</i> : MB 453
<i>Lactobacillus plantarum</i> : MB 452

donors (7 females, mean age: 44 years; 5 males, mean age: 52 years). Blood from the same donor was used for each co-incubation with all tested bacteria in different concentrations. Incubation experiments were repeated 4 to 6 times with blood from different donors. The study was performed in accordance with the Declaration of Helsinki and the local ethics committee.

Separation of PBMNC

PBMNC from healthy donors were separated according to Boyum^[26]. Briefly, peripheral blood diluted with Hank's balanced salt solution (HBSS) (without Ca²⁺ or Mg²⁺) (GIBCO, Karlsruhe, Germany) containing 100 U/mL heparin, was layered over a ficoll (Lymphoprep, Progen, Biotechnik, Heidelberg, Germany; specific gravity: 1.077) and centrifuged for 40 min at 400 r/min without using a frame. Cells harvested from the interface were washed in HBSS (without Ca²⁺ or Mg²⁺) and centrifuged for 10 min at 400 r/min. Supernatant was discarded and the pellet was resuspended in HBSS (without Ca²⁺ or Mg²⁺), which was repeated four times. Finally, resuspension was performed in RPMI 1640 (GIBCO, Karlsruhe, Germany) with 10% fetal calf serum, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 50 μ g/mL gentamycin (all Sigma, St. Louis, MO). Viability of cells was tested by trypan-blue, which was more than 97%. After calculation of the cells per volume, the cell count was adjusted to 500 000 cells per well and per mL.

Bacteria and culture conditions

The bacteria species and strains used in this study are listed in Table 1. Strains in bold type originated from the pharmaceutical probiotic VSL#3 (Sigma Tau, Pomezia, Italy). *Bifidobacterium* and *Lactobacillus* strains were grown in MRS broth (Difco, Detroit, MI) with the addition of 0.05% L-cysteine hydrochloride monohydrate (Merck, Darmstadt, Germany). All strains were incubated anaerobically at 37°C. Mid log cultures counted by plating technique on the above mentioned media, were collected by centrifugation (8000 \times g for 3 min), washed and resuspended in 5 mL RPMI 1640 Medium (GIBCO, Karlsruhe, Germany). The bacterial suspensions were subsequently sonicated (Branson Sonifier W-250, Heinemann, Schwäbisch, Germany) at power levels 5-6 at 30% duty for 5 min to destroy cellular membranes. The sonicated suspension was centrifuged at 8000 \times g for 30 min to separate cell debris from crude cell extract. After centrifugation the supernatant containing the

cell extract and the pellet containing the cell debris were taken for further investigation.

Bacterial incubation with PBMNC

Bacterial cell debris and extract were applied at concentrations ranging from 1×10^3 to 1×10^{10} colony forming units (CFUs)/mL. One hundred μ L at specific concentration was transferred to 900 μ L medium containing 500 000 mononuclear cells and co-incubated at 37°C and 50 mL/L CO₂ for 36 h. Supernatants were collected and stored at -20°C until assay. The viability of PBMNC was checked by the trypan blue test. On each incubation plate a positive control with LPS added to PBMNC and a negative control without stimulus were investigated. The same set-up was used in order to examine the ability of probiotics to inhibit LPS-induced cytokine release. PBMNC were incubated with bacterial cell extract and debris for 10 min, then LPS was added in a concentration of 100 ng/ μ L.

Cytokine quantification in culture supernatant

Cytokine quantification in culture supernatant was analysed by commercially available sandwich enzyme linked immunosorbent assay (ELISA).

TNF- α was detected using anti-human TNF- α monoclonal “capture” antibody (MAB 610, R&D Systems, Minneapolis, MN, USA) and biotinylated “detection” antibody (BAF 210 R&D Systems, Minneapolis, MN, USA) with o-phenylenediamine buffer/H₂O₂ (Sigma, Steinheim, Germany) as substrate. Standard procedure was performed by using recombinant human TNF- α (210-TA, R&D Systems, Minneapolis, MN, USA). The absorbance values of the sample were read at 490 nm on an ELISA plate reader. ELISA measurements were performed in duplicates or triplicates.

IL-1 β was detected using anti-human IL-1 β monoclonal “capture” antibody (MAB 601, R&D Systems, Minneapolis, MN, USA) and biotinylated “detection” antibody (BAF 201 R&D Systems, Minneapolis, MN, USA) with o-phenylenediamine buffer/H₂O₂ (Sigma, Steinheim, Germany) as substrate. Standard procedure was performed by using recombinant human IL-1 β (201-LB, R&D Systems, Minneapolis, MN, USA). The absorbance values of the sample were read at 490 nm on an ELISA plate reader. ELISA measurements were performed in duplicates or triplicates.

IL-10 was detected using anti-human IL-10 “capture” antibody (18551A Pharmingen, San Diego, CA, USA) and biotinylated “detection” antibody (18562D, Pharmingen, San Diego, CA, USA) with o-phenylenediamine buffer/H₂O₂ (Sigma, Steinheim, Germany) as substrate. Standard procedure was performed by using recombinant human IL-10 (19701V, Pharmingen, San Diego, CA, USA, USA). The absorbance values of the sample were read at 490 nm on an ELISA plate reader. ELISA measurements were performed in duplicates or triplicates.

Statistical analysis

Data of cytokine concentration were presented as mean \pm SE and expressed in pg/mL. For quantification of

stimulating capacity of bacteria at different concentrations, cytokine concentration was resumed as an area under the curve (AUC) and described as (mean \pm SE) AUC. Statistical significance was calculated by the Mann-Whitney-Rank test and expressed as *P*-value.

RESULTS

Positive and negative controls

Results from cytokine production in PBMNC after stimulation with LPS (100 ng/mL) were pooled (144 samples from 12 different donors). The mean cytokine production in stimulated PBMNC was 186.5 \pm 125.6 pg/mL for IL-10, 1875.6 \pm 1381.2 pg/mL for IL-1 β and 356.0 \pm 249.1 pg/mL for TNF- α . Cytokine production in non-stimulated PBMNC was under detection limit of the ELISA.

Cytokine production in PBMNC after incubation with cell debris and extract

Generally, cytokine production in PBMNC induced by bacteria differed in cell debris and extract between bacteria and depended on the applied concentration used (Figure 1).

IL-10 concentration

Lactobacilli: Cell extracts from all applied *lactobacilli* induced IL-10 concentration only weakly, whereas no difference was found between species (data not shown). The stimulation by cell debris of these strains led to higher concentrations of IL-10 whereas significance only reached in *L. azidophilus* MB443, *L. delbrueckii* subsp. *bulgaricus* MB453 and *L. plantarum* MB452 (AUC: *L. azidophilus* MB443: 1.0 \pm 1.5 pg/mL; *L. delbrueckii* subsp. *bulgaricus* MB453: 3.8 \pm 4.65 pg/mL; *L. plantarum* MB452: 9.9 \pm 13.8 pg/mL). The stimulation by cell debris of both *L. casei* subs. (MB 451 and *L. GG*) did not differ from that of cell extract of these strains. As shown in Figures 2 and 3, the cell debris of *lactobacilli* had a weak stimulation capacity at concentrations less than 10⁵ CFU/mL.

Bifidobacteria: Cell debris of each *bifidobacteria* strain stimulated IL-10 production in PBMNC more significantly than their cell extracts (AUC: *B. breve* Y8 cell debris: 1062.7 \pm 889.9 pg/mL; cell extract: 182.6 \pm 177.5 pg/mL; AUC *B. longum* Y10 cell debris: 682.7 \pm 466.9 pg/mL; cell extract: 30.5 \pm 28.9 pg/mL; AUC *B. infantis* Y1 cell debris: 1095.6 \pm 925.3 pg/mL; cell extract: 228.3 \pm 233.0 pg/mL; *P* < 0.05) (Figure 1). No difference was found between different *bifidobacteria* species when the stimulation capacity of cell extracts or cell debris was compared. Cell debris from *bifidobacteria* stimulated IL-10 production in PBMNC more significantly than *lactobacilli* (AUC cell debris of *bifidobacteria*: 710.1 \pm 795.5 pg/mL; AUC cell debris of *lactobacilli*: 219.6 \pm 174.7 pg/mL; *P* < 0.02) (Figure 4).

As shown in Figure 2 and Figure 3, the cell debris of *bifidobacteria* had a weak stimulating capacity at the concentration lower than 10³ CFU/mL. Moreover, the highest concentration (10⁸ CFU/mL) of *bifidobacteria* had a less stimulating capacity than the lower concentration (10⁷ CFU/mL), whereas the viability measured by trypan blue test was over 97%.

E. coli nissle: Cell extracts and debris of *E. coli* nissle led

to similar IL-10 concentrations (AUC cell debris: 1270.7 ± 210.9 pg/mL; cell extract: 1154.8 ± 264.0 pg/mL) (Figure 1). The stimulation ability of cell extract to produce IL-1 β in PBMNC was significantly higher in *E. coli* nissle than in *lactobacilli* or *bifidobacteria* ($P < 0.05$). The cell debris of *E. coli* nissle had a similar stimulating capacity to *bifidobacteria* but a more significant capacity than *lactobacilli* ($P < 0.02$) (Figure 4). The cell debris and extract of *E. coli* nissle even had a stimulating capacity at low concentrations (Figure 3). The highest concentration (10^8 CFU/mL) of *E. coli* nissle had a less stimulating capacity than the lower concentration (10^7 CFU/mL), whereas the viability measured by trypan blue test was over 97%.

IL-1 β concentration

Lactobacilli: As shown in Figure 1 cell extracts from all applied *lactobacilli* induced IL-1 β concentration only weakly, whereas no difference was found between species (data not shown). The stimulation by cell debris of *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus* and *L. plantarum* led to higher concentrations of IL-1 β (AUC: *L. azidophilus* MB443: 1282.2 ± 987.9 pg/mL; *L. delbrueckii* subsp. *bulgaricus* MB453: 4881.5 ± 893.8 pg/mL; *L. plantarum* MB452: 3390.8 ± 288.9 pg/mL) ($P < 0.05$) (Figure 1). The stimulation by cell debris did not differ from cell extract of *L. casei* subs. *rhamnosus* (L.GG) and *L. casei* MB451. As shown in Figures 2 and 3, the cell debris of *lactobacilli* had only a weak stimulating capacity at concentrations of less than 10^6 CFU/mL.

Bifidobacteria: Cell debris of each *bifidobacteria* strain stimulated IL-1 β production in PBMNC more significantly than their cell extract (AUC: *B. breve* Y8 cell debris: 11152.9 ± 2547.7 pg/mL; cell extract: 1488.3 ± 454.0 pg/mL; AUC *B. longum* Y10 cell debris: 12364.0 ± 192.5 pg/mL; cell extract: 491.0 ± 190.6 pg/mL; AUC *B. infantis* Y1 cell debris: 9018.8 ± 2190 pg/mL; cell extract: 2142.3 ± 925.0 pg/mL; $P < 0.05$) (Figure 1). No difference was found between *bifidobacteria* species when the stimulating capacity of cell extracts or cell debris was compared. The cell debris from *bifidobacteria* led to higher IL-1 β concentrations in supernatant of PBMNC than cell debris of *lactobacilli* (AUC cell debris of *bifidobacteria*: 11692.8 ± 2283.2 pg/mL; AUC cell debris of *lactobacilli*: 3143.3 ± 2689.0 pg/mL; $P < 0.02$) (Figure 4). As shown in Figure 2, the cell debris of *B. breve* Y8 and *B. longum* Y10 even had a stimulating capacity at low concentrations (10^3 CFU/mL and 10^2 CFU/mL), whereas the IL-1 β production in PBMNC was weak when incubated with *B. infantis* Y1 at a concentration of less than 10^4 CFU/mL. The highest cell debris concentration (10^8 CFU/mL) of *bifidobacteria* had a less stimulating ability to produce IL-1 β in PBMNC than its lower concentration, whereas the viability measured by trypan blue test was over 97%.

E. coli nissle: Cell extracts and debris of *E. coli* nissle led to similar IL-1 β concentrations (AUC cell debris: 9334.8 ± 2486.1 pg/mL; cell extract: 7875.0 ± 1595.3 pg/mL) (Figure 1). The stimulating ability of the cell extract of *E. coli* nissle to produce IL-1 β in PBMNC was significantly higher than that of *lactobacilli* or *bifidobacteria* ($P < 0.05$). The cell debris of *E. coli* nissle had a similar stimulating

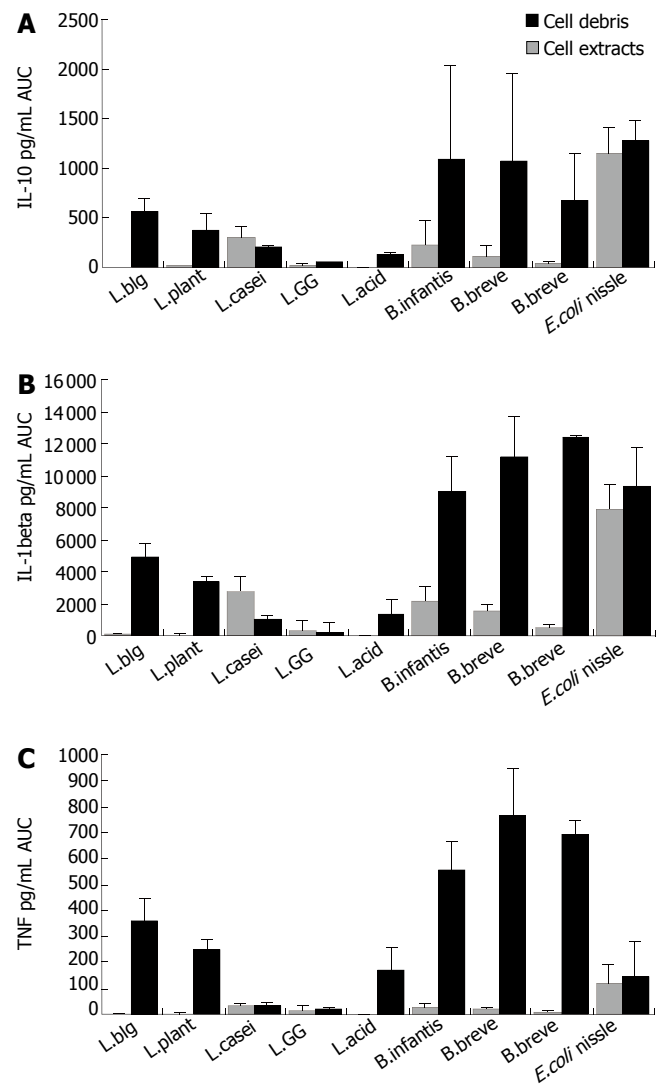


Figure 1 Concentration of IL-10 (A), IL-1 β (B) and TNF α (C) by PBMNC (area under the curve of mean \pm SE) after incubation with cell debris (■) or cell extract (▒) of different strains.

capacity to *bifidobacteria* but a more significant capacity than *lactobacilli* ($P < 0.02$) (Figure 4). The cell debris and extract of *E. coli* nissle even had a stimulating capacity at low concentrations (Figure 3). The highest concentration of debris of *E. coli* nissle had a less stimulatory capacity than lower concentration.

TNF-concentration

Lactobacilli: Cell extracts from *L. azidophilus* MB443, *L. delbrueckii* subsp. *bulgaricus* MB453, and *L. plantarum* MB452 induced TNF- α concentration only weakly. The stimulation by cell debris of these strains led to higher TNF- α production (AUC: *L. azidophilus* MB443: 1695.3 ± 879.3 pg/mL; *L. delbrueckii* subsp. *bulgaricus* MB453: 3593.2 ± 822.1 pg/mL; *L. plantarum* MB452: 2466.5 ± 433.3 pg/mL) ($P < 0.05$) (Figure 1). The stimulation by cell debris did not differ from cell extract of *L. casei* subs. *rhamnosus* (L.GG) and *L. casei* MB451. As shown in Figures 2 and 3, the cell debris of *lactobacilli* only had a weak stimulating capacity at concentrations of less than 10^7 CFU/mL.

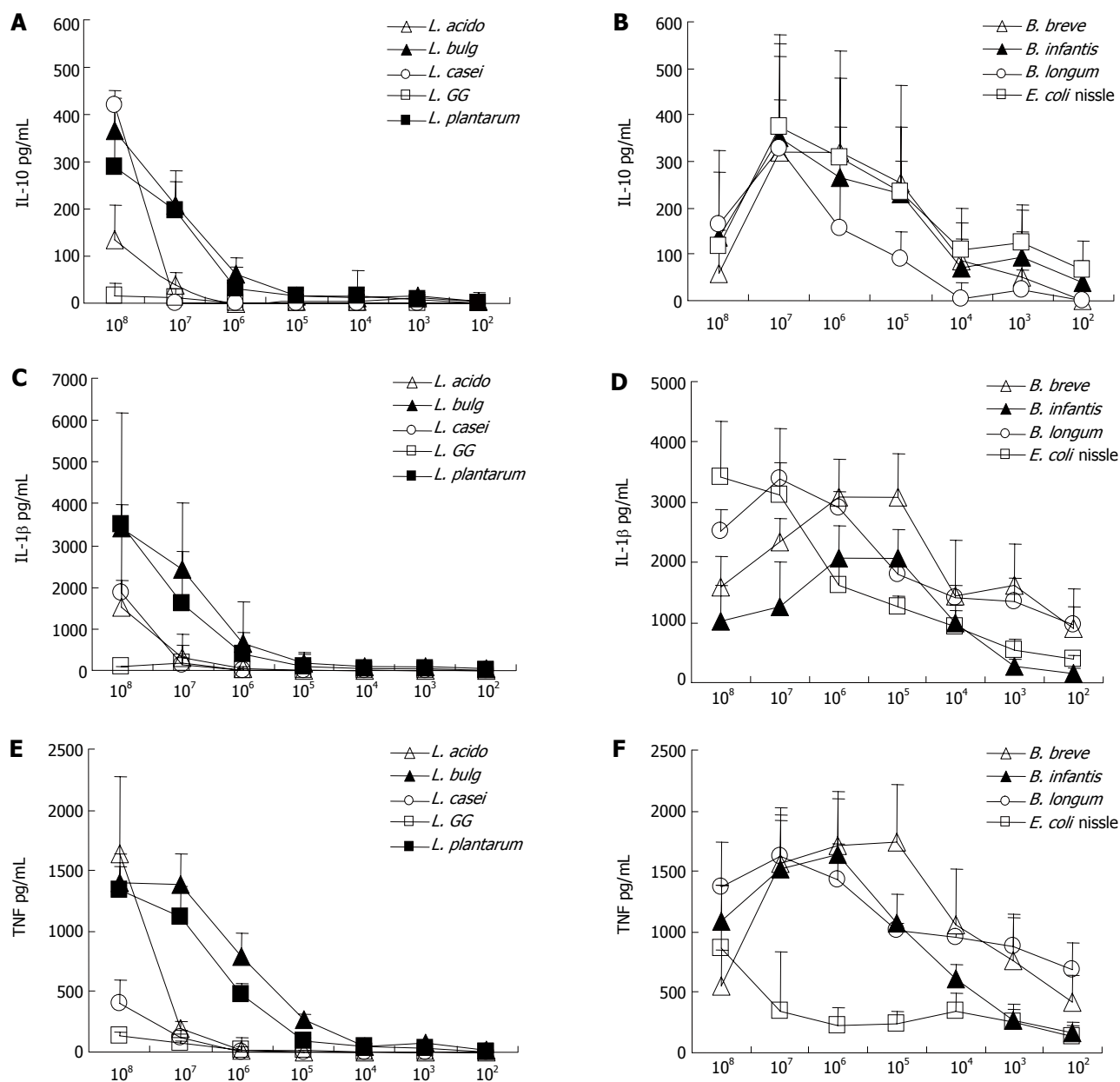


Figure 2 Cytokine concentration of supernatant after incubation of PBMC with cell debris of bacteria in different concentrations. **A** and **B**: supernatant concentration of IL-10 in pg/ml (mean \pm SE); **C** and **D**: supernatant concentration of IL-1 β in pg/ml (mean \pm SE); **E** and **F**: supernatant concentration of TNF α in pg/mL (mean \pm SE). *Lactobacilli* are described on the left side and *bifidobacteria* are described on the right side.

Bifidobacteria: Cell debris of each *bifidobacteria* strain led to higher TNF α concentrations in supernatant of PB-MNC than their cell extract (AUC: *B. breve* Y8 cell debris: 7645.5 \pm 1823.1 pg/mL; cell extract: 180.1 \pm 85.5 pg/mL; AUC *B. longum* Y10 cell debris: 6951.2 \pm 522.7 pg/mL; cell extract: 83.6 \pm 40.0 pg/mL; AUC *B. infantis* Y1 cell debris: 5584.8 \pm 1098.8 pg/mL; cell extract: 290.5 \pm 103.6 pg/mL) ($P < 0.05$). No difference was found between *bifidobacteria* species when the stimulating capacity of cell extract or cell debris was compared. The cell debris from *bifidobacteria* led to higher TNF α concentrations in supernatant of PB-MNC than cell debris from *lactobacilli* or *E. coli* nissle (AUC cell debris of *bifidobacteria*: 6882.4 L \pm 1355.0 pg/mL; AUC cell debris of *lactobacilli*: 1630.8 \pm 1265.1 pg/mL; AUC cell debris of *E. coli* nissle: 1466.6 \pm 1356.0 pg/mL) ($P < 0.02$)

(Figure 4). As shown in Figure 2, the cell debris from *B. breve* Y8 and *B. longum* Y10 even had a stimulating capacity at low concentrations (10^3 CFU/mL and 10^2 CFU/mL), whereas the TNF- α production in PBMC was weak when incubated with *B. infantis* Y1 at a concentration of less than 10^4 CFU/mL. The highest concentration (10^8 CFU/mL) of *bifidobacteria* had no strong stimulating ability to produce TNF- α in PBMC, whereas the viability measured by trypan blue test was over 97%.

***E. coli* nissle:** High concentrations (10^8 CFU/mL) of cell debris of *E. coli* nissle led to high concentrations of TNF- α , whereas lower concentrations of cell debris and extracts led to lower concentrations of TNF- α (Figure 3) (AUC cell debris: 1466.6 \pm 1356 pg/mL; cell extract: 1153.0 \pm 748.4 pg/mL) The cell debris of *E. coli* nissle

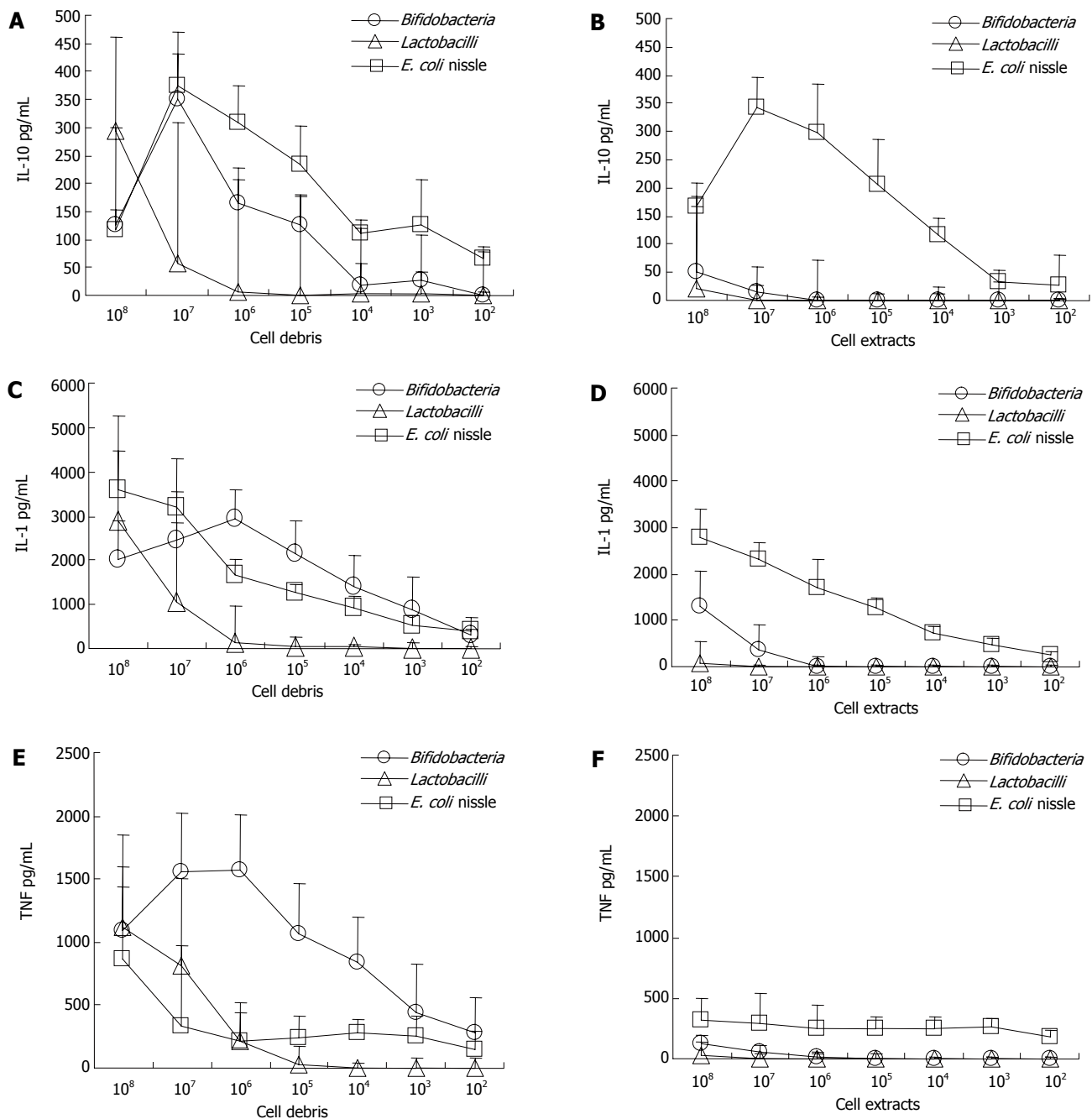


Figure 3 Cytokine concentrations of supernatant after incubation of PBMC with cell debris (left side) or cell extracts (right side) of bacteria from different species families in different concentrations. **A** and **B**: supernatant concentration of IL-10 in pg/ml (mean \pm SE); **C** and **D**: supernatant concentration of IL-1 β in pg/ml (mean \pm SE); **E** and **F**: supernatant concentration of TNF α in pg/mL (mean \pm SE).

had a similar stimulating capacity similar to *lactobacilli* but a significantly less capacity than that of *bifidobacteria* ($P < 0.02$) (Figure 4).

Inhibition of stimulated PBMC by bacterial cell debris and extract

None of the bacterial cell debris and extract of *lactobacilli*, *bifidobacteria* or *E. coli nissle* had an inhibitory effect on the cytokine production in PBMC. Pre-incubation with cell debris or with cell extract and stimulation with LPS led to similar cytokine production of LPS alone or cell debris alone (data not shown).

DISCUSSION

Different probiotic strains used in clinical trials have shown prophylactic properties in different inflammatory diseases of the gastrointestinal tract, such as Crohn's disease, ulcerative colitis, pouchitis, antibiotic-associated colitis and traveller's diarrhoea^[27-28]. Recently, we have shown that the IL-10 concentration in the mucosa of ileo- anal pouch tissue is elevated after administration of probiotics^[25]. In this *in vivo* approach we used a highly concentrated probiotic preparation containing different lactic acid bacteria. Our hypothesis is that a high concentration of bacteria contributes to these clinical results and

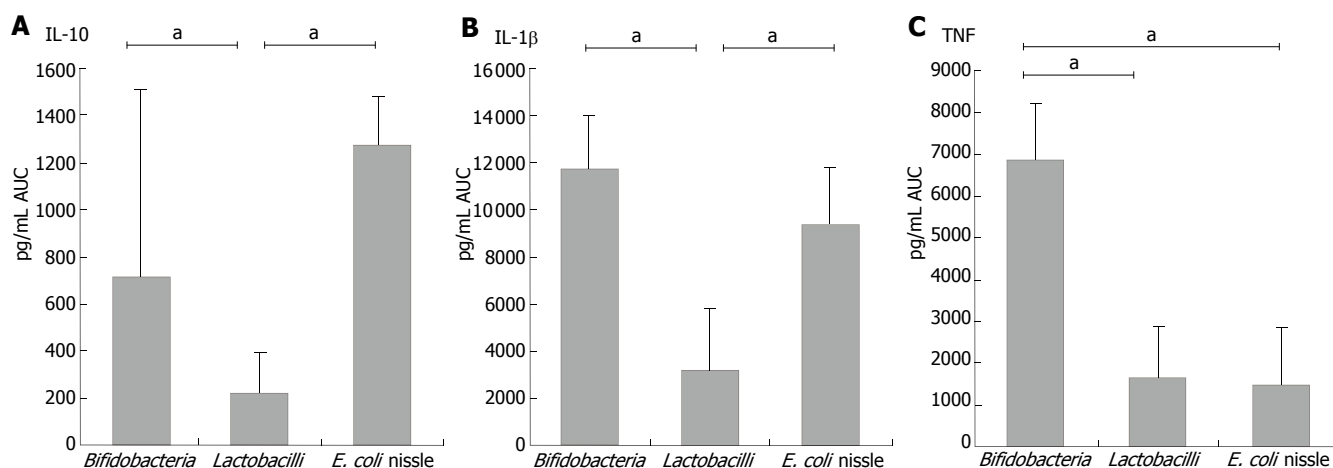


Figure 4 Supernatant concentrations of different cytokines after co-incubation of PBMC with cell debris of bacteria from different species families. With regard to *lactobacilli* results are pooled from *L.acidophilus*, *L.bulgaricus*, *L.casei*, *L.GG*, *L.plantarum*. With regard to *bifidobacteria* results are pooled from *B.breve*, *B.infantis*, *B.longum*. IL-10, IL-1β and TNFα shown as area under the curve (mean ± SE) ($^{\circ}P < 0.05$).

immunologic findings^[3]. However, the specific property of different strains remains unclear. Therefore, we investigated an *in vitro* model in order to test different probiotic strains and species, which are used in clinical practice to prevent inflammatory diseases, in order to understand the pro- and anti-inflammatory properties of specific strains. There are several studies on the induction of cytokines by cell components of lactic acid bacteria to induce cytokines^[29-31]. However, systematic analysis of probiotic bacteria used in clinical practice for the prevention of inflammatory disease, has not been performed until now in a human cell model. For this purpose we used PBMC which are easily available and express toll-like-receptor (TLR) 2 and TLR 4 as well as CD14 which are shown to mediate immune response to microbial components as peptidoglycan and lipoteichoic acid^[32-33]. Until now there is no report on comparison of dose response over a broad range of different concentrations of probiotic bacteria used in clinical practice for prevention of inflammatory bowel disease. Our findings on PBMC indicate that stimulation by *lactobacilli* works in a dose-dependent way. High doses of cell debris could stimulate PBMC to produce pro- and anti-inflammatory cytokines. The cell extract has a less stimulating capacity in a dose-dependent manner. An interesting finding is that the cell debris of *L. delbrueckii* subsp. *bulgaricus* MB453 and *L. plantarum* MB452 stimulates PBMC when used at concentrations higher than 10^4 CFU/mL, while both *L. azidophilus* MB443 and *L. casei* MB451 strains only require concentrations higher than 10^6 and 10^5 CFU/mL. Cell debris of *L. casei* subsp. *rhamnosus* (*L.GG*) had a very low stimulating capacity compared to other strains (Figure 1). The weak or even absent reaction at high concentrations of *lactobacilli* (10^6 or 10^5 CFU/mL) is not suspected. This phenomenon is reproducible in different blood donors and exists in all different *lactobacilli* strains when used for this examination. Schultz and co-workers^[34] recently showed that *L. casei* subsp. *rhamnosus* (*L.GG*), which had the lowest stimulating capacity in our study, induces immunologic tolerance to granulocytes after oral administration for several weeks. Since *lactobacillus*

strains normally are early inhabitants of the human gastrointestinal tract, oral tolerance to low concentrations of *lactobacillus* strains might generally develop^[35]. Although this is expected for *bifidobacteria*, they stimulate pro- and anti-inflammatory cytokines more significantly than *lactobacilli*. But the stimulation pattern is different. The highest concentration of *bifidobacteria* induces PBMC to produce less pro- and anti-inflammatory cytokines than lower concentrations of the strain. Whether the lower induction of cytokine release in incubation with highly concentrated cell debris is due to deletion or apoptosis of PBMC remains unclear. Toxic reaction or a reaction resulting in direct cell death can be excluded by the trypan blue control test which provides information about the functionality of cell membrane but not about the metabolic condition of cells. Recently, it has been proposed in a different model that *bifidobacteria* strains induce oral tolerance^[36] but also induction of oral tolerance to *E. coli* and *lactobacilli* has been reported^[37-38]. The stimulating capacity of *E. coli* nissle shows a different pattern. The cell extract and debris of *E. coli* nissle have a similar ability to produce cytokines. Interestingly, the cell debris and extract of *E. coli* nissle at low concentrations can stimulate epithelial HT29/19 cells to produce the chemotactic factor interleukin-8 (IL-8), whereas the cell debris and extract of *lactobacilli* and *bifidobacteria* do not stimulate epithelial HT29/19 cells to release IL-8^[38]. *E. coli* nissle, which has been shown to be effective in maintaining remission of ulcerative colitis, has a high stimulating capacity for IL-10 and IL-1β compared to other strains, but a low capacity for TNF-α. *Bifidobacteria* of the probiotic composition VSL#3 of *bifidobacteria*, which can prevent inflammatory bowel disease, can stimulate PBMC to produce IL-10^[9,10]. *L. GG* can weakly stimulate PBMC to produce IL-10 and has no positive effect on inflammatory bowel disease^[7-8]. This is consistent with the findings in another study^[25]. *L. GG* has been primarily used in trials for prevention of relapses in Crohn's disease. It has been recently reported that Crohn's disease is associated with the polymorphism of the nucleotide-binding oligomerization domain 2

(NOD 2)^[39-40]. NOD 2 is a regulator of TLR 2-mediated response to microbial agents^[41] and Gram-positive bacteria like *lactobacilli* are typical ligands for TLR 2^[32]. Since the function of mutations in the NOD 2 gene in Crohn's disease is not clear^[42], explanation about the lacking effect of probiotics on Crohn's disease is warranted.

In conclusion, the ability of probiotic bacteria to stimulate PBMNC is different. Compared to *E. coli* nissle and *bifidobacteria*, *lactobacilli* debris exerts effects only at high concentrations. Whereas the extract of *lactobacilli* and *bifidobacteria* has only weak effects, while the cell extract and debris of *E. coli* nissle have similar effects. The higher IL-10 response to *E. coli* nissle and *bifidobacteria* corresponds to the positive effect of these probiotic strains on inflammatory bowel disease compared to negative outcomes obtained with *lactobacilli*.

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Effects of probiotic bacteria on gastrointestinal motility in guinea-pig isolated tissue

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Abstract

AIM: To evaluate the intestinal motility changes evoked by 8 bacterial strains belonging to *Bifidobacterium*, *Lactobacillus* and *Streptococcus* genera within the probiotic preparation VSL#3.

METHODS: Ileum and proximal colon segments isolated from guinea-pigs were used as a study model. Entire cells and cell fractions (cell debris, cell wall fraction, cytoplasmatic fraction, proteinaceous and non-proteinaceous cytoplasmatic components) of VSL#3 strains and, as controls, *Escherichia coli*, *Salmonella aboni* and *Bacillus licheniformis* were tested in this *in vitro* model.

RESULTS: Among the bacterial cell fractions tested, only the cytoplasmatic fraction modified intestinal motility. *Lactobacillus* strains stimulated the contraction of ileum segment, whereas all probiotic strains tested induced proximal colon relaxation response. The non-proteinaceous cytoplasmatic components were responsible for the colon relaxation.

CONCLUSION: The results obtained in this study suggest that the proximal colon relaxation activity showed by the probiotic bacteria could be one of the possible mechanisms of action by which probiotics exert their positive effects in regulating intestinal motility.

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Key words: Probiotics; Intestinal motility; Guinea-pigs; Ileum; Colon

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INTRODUCTION

Probiotics are viable microbial cells that upon ingestion in specific numbers appear to have beneficial effects on the health and well-being of the host, beyond inherent basic nutrition^[1]. These health-promoting effects are predominantly related to reinforcement of the intestinal mucosal barrier against enteropathogens' colonization, immunostimulation and immunomodulation, anticarcinogenic and antimutagenic activities, improvement of lactose utilization, and reduction of serum cholesterol^[2].

Most probiotics are bacteria members of the genera *Lactobacillus* and *Bifidobacterium*, which represent important components of human gastrointestinal flora. However, other nonpathogenic bacteria, such as *Streptococcus*, some strains of *Escherichia coli* and *Enterococcus faecium*, and yeasts, such as *Saccharomyces boulardii*, have been used in probiotic preparations^[3].

Experimental and clinical studies support the use of probiotics in the treatment of intestinal disorders such as infectious diarrhea^[4-7], antibiotic diarrhea^[8-10], traveller's diarrhea^[11-13], irritable bowel syndrome or functional diarrhea^[14-18] and inflammatory bowel disease^[19-23]. In recent double-blind placebo controlled trials the efficacy of the probiotic preparation VSL#3 has been shown as maintenance treatment and prophylactic therapy in patients with diarrhea-predominant irritable bowel syndrome and pouchitis^[18,22-24]. VSL#3 (VSL Pharmaceuticals, Ft. Lauderdale, FL, USA) contains a mixture of eight different bacterial species at very high concentrations (450 billions/sachet of viable lyophilized bacteria). The preparation consists of three strains of *bifidobacteria* (*Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium infantis*), four strains of *lactobacilli* (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus plantarum*) and one strain of *Streptococcus thermophilus*.

To the best of our knowledge, there are no experimental data evaluating the effects of probiotic bacteria on

the intestinal motility. Functional assays could represent useful tools to investigate the effects evoked by the entire probiotic cells and/or their fraction on different segments of the intestinal tissue.

The aim of this study was to evaluate the effect of the eight strains within the probiotic preparation VSL#3^[18,21-23] on intestinal motility using ileum and proximal colon segments isolated from guinea-pigs. Entire bacterial cells, cell fractions of VSL#3 strains, and, as controls, *Escherichia coli*, *Salmonella aboni* and *Bacillus licheniformis*, were tested in this *in vitro* model.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The following bacterial strains were used in this study: *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus plantarum* and *Streptococcus thermophilus*.

Bifidobacterium and *Lactobacillus* strains were grown anaerobically (Anaerobic System, Model 2028, Forma Scientific, Marietta, OH, USA) in MRS medium (Difco, Detroit, MI, USA) supplemented with 0.05% L-cysteine hydrochloride monohydrate (Merck, Darmstadt, Germany), at 37°C. *S. thermophilus* was cultured anaerobically in M17 medium (Difco) at 37°C. *Salmonella aboni* NCTC 6017, *Escherichia coli* ATCC 11105 and *Bacillus licheniformis* BGSC 5A24 were used as controls and grown aerobically in LB medium (Difco) at 37°C.

Preparation of bacterial cell fractions

Ten milliliter of the bacterial mid log cultures, counted by plating technique on the previously mentioned media, was collected by centrifugation ($5000 \times g$ at 4°C for 5 min), washed, and resuspended in 1 mL of De Jalon buffer (155 mmol/L NaCl, 5.6 mmol/L KCl, 0.5 mmol/L CaCl₂, 6.0 mmol/L NaHCO₃, 2.8 mmol/L glucose)^[25], obtaining a final bacterial concentration of 1×10^9 - 1×10^{10} colony forming units (CFUs/mL). Subsequently the bacterial suspensions were sonicated (Bronson Sonifier W-250, Heineman, Schwäbisch, Germany) at power level 2 at 20% duty for 8 min and centrifuged at $8000 \times g$ for 30 min to separate cell debris from crude extract. Cell debris was resuspended in 1 mL of De Jalon buffer. The crude extract was ultracentrifuged at $250\,000 \times g$ at 4°C for 2 h: the supernatant represents the cytoplasmatic fraction while the pellet, resuspended in 1 mL of 50 mmol/L TRIS.HCl pH 7.6, represents the fraction enriched in membrane proteins.

In order to obtain the bacterial cell wall fraction, bacterial cultures were centrifuged at $5000 \times g$ at 4°C for 5 min, washed in 50 mmol/L Tris HCl pH 7.6, resuspended in 1 mL of protoplast buffer [50 mmol/L Tris HCl pH 7.6, 1 mol/L sucrose, 50 mL of completeTM protease inhibitor (Roche, Mannheim, Germany), 15 mg/mL lysozyme] and incubated at 37°C for 20 min. The supernatant was recovered by centrifuging at $5000 \times g$ at 4°C for 5 min.

Proteinaceous components were precipitated by addition of 9 volumes of acetone:HCl (10:0.1) to one

volume of the cytoplasmatic fraction and collected by centrifuging at $12000 \times g$ at 4°C for 5 min. Supernatant (non-proteinaceous fraction) and protein pellet were dried on ice under vacuum for removing acetone and resuspended in the initial volume of 50 mmol/L TRIS.HCl pH 7.6. The very low value of pH (about 1) of the non-proteinaceous fraction was adjusted to a value of 7.4 to overcome the unspecific colonic contractile response due to the acid pH. Similarly, the cytoplasmatic fraction was incubated with proteinase K (500 mg/mL) at 50°C for 1 h for the enzymatic digestion of the proteins. All the bacterial fractions of cell debris (i.e., cell wall fraction, membrane proteins, cytoplasmatic fractions, proteinaceous and non-proteinaceous cytoplasmatic components) were aliquoted and stored at -80°C before to be used in the *in vitro* stimulation of ileum and proximal colon.

SDS PAGE

Ten μ L of *B. infantis* cytoplasmatic fraction, non-proteinaceous fraction and cytoplasmatic fraction deprived of proteins by proteinase K digestion was analyzed by SDS-PAGE, as described by Laemmli^[26], using a 12% polyacrylamide running gel. The gels were stained with silver nitrate.

Bacterial DNA preparation

Isolation of genomic DNA from pure cultures of the probiotic bacteria was performed as previously described^[27]. In order to obtain the complete cell disruption, the method was slightly modified by prolonging the enzymatic lysis for 1 to 3 h and grinding with glass beads (150-212 μ m, Sigma, St. Louis, MO, USA). Concentration and purity of all DNA preparations were determined by measuring OD₂₆₀ absorbance and OD_{260/280} ratio, respectively. Only DNAs with an OD_{260/280} ratio > 1.8 were used.

Animals and preparation of Guinea-pig ileum and proximal colon

Guinea-pigs of either sex (200-400 g) obtained from Charles River (Calco, Como, Italy) were used. The animals were housed according to the ECC Council Directive regarding the protection of animals used for experimental and other scientific purposes. All procedures followed the guidelines of The Animal Care and Use Committee of The University of Bologna (Bologna, Italy). The animals were sacrificed by cervical dislocation, and the organ (ileum or proximal colon) required was set up rapidly under a suitable resting tension in 15-mL organ baths containing appropriate physiological salt solution (PSS) consistently warmed (see below) and buffered to pH 7.4 by saturation with 950 mL/L O₂ and 50 mL/L CO₂ gas.

Preparation of ileum

The terminal portion of ileum (3-4 cm near the ileo-caecal junction) was cleaned with Tyrode solution of the following composition: 118 mmol/L NaCl, 4.75 mmol/L KCl, 2.54 mmol/L CaCl₂, 1.20 mmol/L MgSO₄, 1.19 mmol/L KH₂PO₄·2H₂O, 25 mmol/L NaHCO₃, and 11 mmol/L glucose. The mesenteric tissue was removed. The ileum tissue was cut in two segments of 2-3 cm taken in the lon-

gitudinal direction along the intestinal wall. The segments were set up upright under 1-g tension in a jacketed tissue bath (15 mL, 37°C) containing Tyrode solution buffered to pH 7.4 by saturation with 950 mL/L O₂ and 50 mL/L CO₂ gas. Tissues were allowed to equilibrate for at least 30 min, during which time the bathing solution was changed every 10 min. After stabilization, the strips were challenged with 1 µmol/L carbachol (Sigma, Italy) to assess the responsive capacity of the tissue^[28].

Preparation of proximal colon

Starting approximately 1 cm distal from the caecocolonic junction, a segment of about 3 cm was excised, cleansed by rinsing it with De Jalon solution^[25], and the mesenteric tissue was removed. The proximal colon segment was cut in two segments of about 1 cm each taken in the longitudinal direction along the intestinal wall. The segments were set up upright under 1-g tension at 37°C in a jacketed tissue bath (15 mL, 37°C) containing De Jalon solution buffered to pH 7.4 by saturation with 950 mL/L O₂ and 50 mL/L CO₂ gas. Tissues were allowed to equilibrate for at least 30 min during which time the bathing solution was changed every 10 min. After stabilization, the strips were challenged with 5 µmol/L 5-hydroxytryptamine (5-HT) (Sigma, Italy) in the presence of 1 µmol/L atropine (Sigma, Italy) to assess the responsive capacity of the tissue.

Functional assays on ileum and proximal colon

After stabilization and assessment of the responsive capacity of the tissue in the organ bath, concentration-response curves were constructed by cumulative addition of the above described bacterial preparations (1 to 1500 µL). Each successive addition of bacterial preparations was performed after the response to the previous addition reached its maximum level and remained steady. Longitudinal muscle contractions or relaxations were recorded isometrically by securing one end of the tissue segments to a tissue holder and the other end to a force displacement transducer (FT. 03, Grass Instruments, Quincy, MA) using Power Lab software (ADInstruments Pty Ltd, Castle Hill, Australia). Each tracing was obtained by using separate intestinal strips.

Statistical analysis

Experiments were performed in duplicate with tissue from the same animal, and mean values were recorded. All data are presented as mean ± SE ($n = 3-5$ for ileum and $n = 5-7$ for proximal colon). Differences between means were calculated with Student *t*-test and *P*-values < 0.05 were considered statistically significant^[29-31].

RESULTS

Bacterial effect on motility response in guinea-pig ileum

The motility response of the guinea-pig ileum segment was investigated with increasing concentrations of the following components of the probiotic VSL#3 mixture: live bacteria, bacterial cell debris, and crude extracts. VSL#3 live bacteria and cell debris did not modify the guinea-pig ileum motility (data not shown), whereas crude

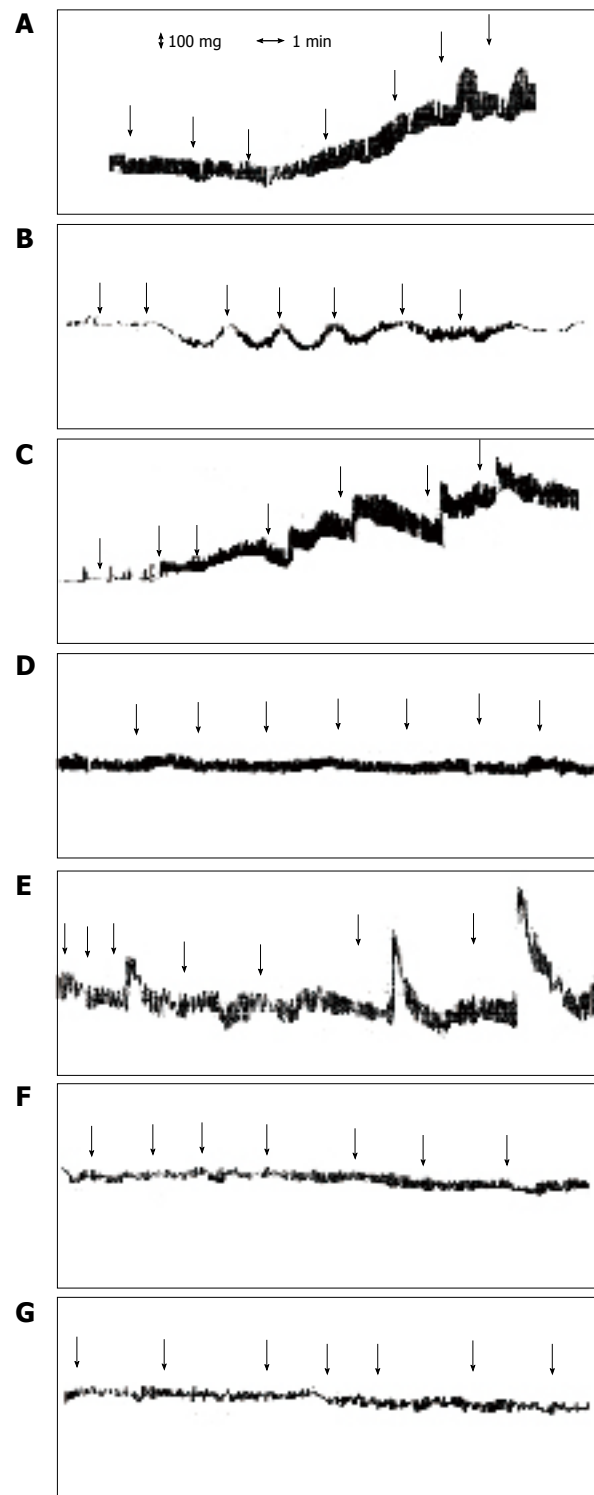


Figure 1 Recorder tracing of the cumulative dose of VSL#3 (A), *Bifidobacterium* (B), *Lactobacillus* (C), *Streptococcus thermophilus* (D), *Salmonella aboni* (E), *Escherichia coli* (F), and *Bacillus licheniformis* (G) crude extracts on the contractility of Guinea-pig isolated ileum. Arrows indicate the addition of 50, 100, 150, 200, 250, 300 and 350 µL of each crude extract tested.

extracts dose-dependently increased both spontaneous phasic and tonic contractions of the ileum (Figure 1A). The effects on motility induced by crude extracts persisted even after adjusting the pH of the solution from around 5.0, as found in the crude extract, to pH 7.4 and were reversible as the basal tone returned to initial values after

several washing steps (data not shown).

To identify the microbial genera within the VSL#3 cocktail responsible for the effects on motility in the guinea-pig ileum, crude extract mixtures of *Bifidobacterium* (*B. longum*, *B. infantis*, *B. breve*), *Lactobacillus* (*L. acidophilus*, *L. casei*, *L. plantarum*, *L. bulgaricus*), and *Streptococcus thermophilus* were individually tested. The following differences in the motility response by the crude extracts of the three probiotic genera were observed: (1) *Lactobacillus* strains induced a concentration-dependent contraction of the ileum, which was rapid in onset; (2) *Bifidobacterium* promoted a weak and transient stimulation of the ileum in a non concentration-dependent manner; (3) *Streptococcus* did not promote any significant response (Figures 1B-D).

The intestinal bacteria *Salmonella aboni* and *Escherichia coli* and the non-intestinal bacterium *Bacillus licheniformis* were used as controls to verify the specificity of the probiotic effects on gut motility (Figures 1E-G). Ileum motility was not affected by the exposure to *E. coli* and *B. licheniformis* crude extracts (Figures 1F, G), whereas *S. aboni* crude extracts were able to trigger the ileum contraction in a dose-dependent manner (Figure 1E). Furthermore, the amplitude of contraction induced by *S. aboni* was significantly higher than that promoted by the VSL#3 mixture and *Lactobacillus* crude extracts.

In order to verify the involvement of muscarinic receptors in the observed contraction response, the muscarinic antagonist atropine (1 $\mu\text{mol/L}$) was added to the organ bath. Atropine did not modify the motility response of ileal tissue exposed to all the bacterial crude extracts tested (data not shown).

Bacterial effect on motility response in guinea-pig proximal colon

The motility response of guinea-pig proximal colon segment was investigated using a similar protocol as with the guinea-pig ileum by adding identical concentrations of the bacterial cell preparations tested in the ileum stimulation study. Similar to the guinea-pig ileum, only the crude extracts of the probiotic VSL#3 mixture showed a significant effect on colon motility. However, the effect on the guinea-pig proximal colon was opposite to that observed in ileum (Figure 2A). In particular, the crude extract of the VSL#3 mixture elicited a rapid and sustained relaxation of the colon tissue, characterized by peaks in response to each addition, together with a progressive lowering of the basal tone. As previously carried out with the ileum, crude extract preparations at pH 5 and 7.4 were tested obtaining the same colon relaxation response (data not shown).

The VSL#3 extract mixtures of the genera *Bifidobacterium*, *Lactobacillus*, and *S. thermophilus* were assessed (Figures 2B-D). All the bacterial groups provoked a dose-dependent relaxation of the colon tissue, but no significant motility effect was observed by exposure to the control bacteria (Figures 2E-G). Clear colon relaxation effects were promoted by the crude extracts of *Bifidobacterium* strains and *S. thermophilus* and were quite similar to those induced by the crude extracts of the VSL#3 mixture (Figure 3). *Lactobacillus* strain's crude extract showed a lower relaxation

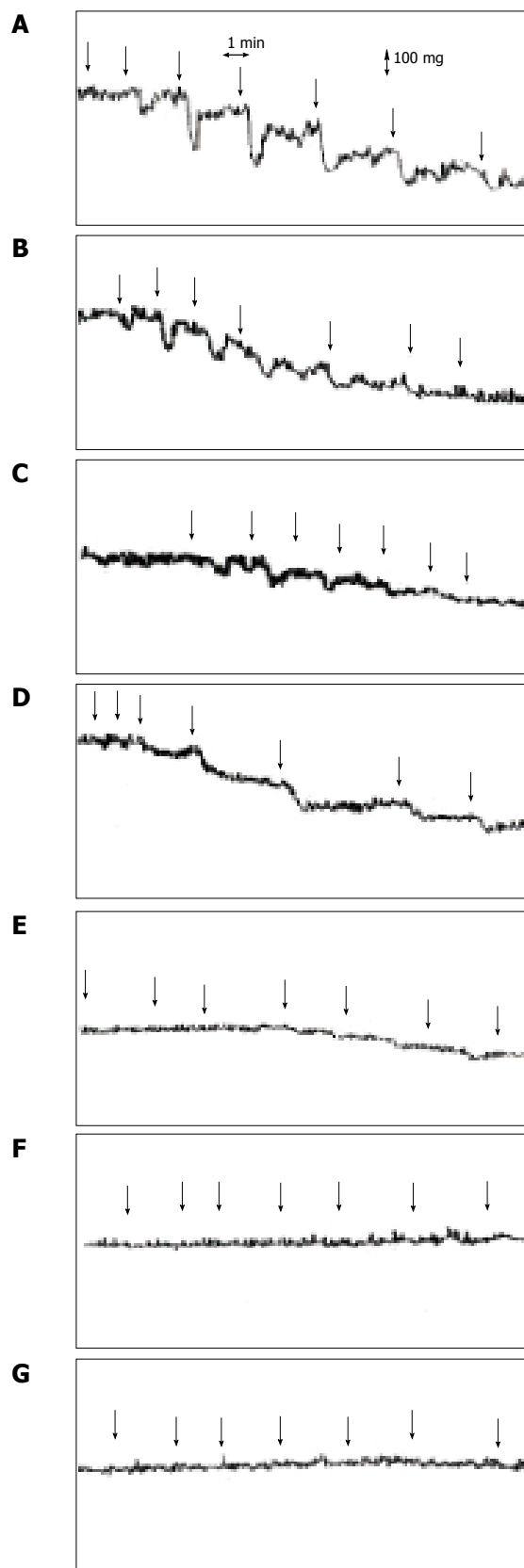
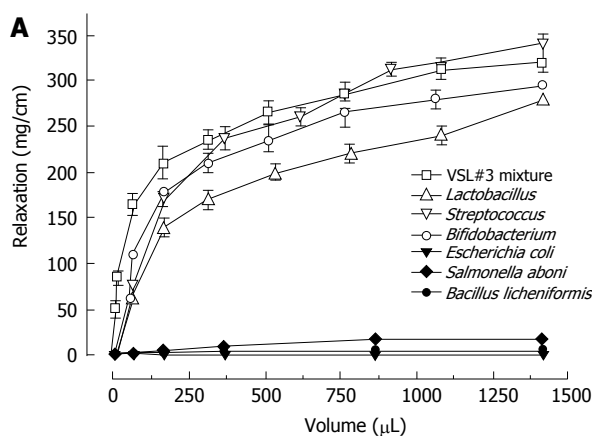


Figure 2 Recorder tracing of the cumulative dose of VSL#3 (A), *Bifidobacterium* (B), *Lactobacillus* (C), *Streptococcus thermophilus* (D), *Salmonella aboni* (E), *Escherichia coli* (F), and *Bacillus licheniformis* (G) crude extracts on the contractility of Guinea-pig isolated proximal colon. Arrows indicate the addition of 50, 100, 150, 200, 250, 300 and 350 μL of each crude extract tested.

response. Washings of the colon segment exposed to



Bacterial strains	μL	Relaxation %
VSL#3 mixture	1100	100
<i>Lactobacillus</i>	1400	86 ± 3.9
<i>Streptococcus</i>	1400	106 ± 7.5
<i>Bifidobacterium</i>	1050	92 ± 1.3
<i>Escherichia coli</i>	1400	0
<i>Salmonella aboni</i>	1400	0
<i>Bacillus licheniformis</i>	1400	6 ± 0.4

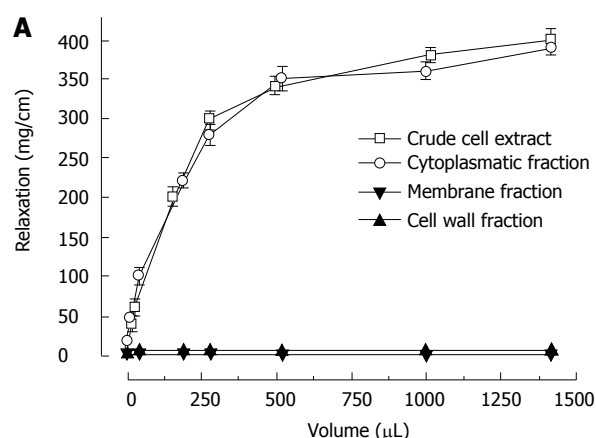
Figure 3 Relaxation induced by crude extract of VSL#3 mixture, *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, *Escherichia coli*, *Salmonella aboni* and *Bacillus licheniformis* preparations on Guinea-pig proximal colon. Relaxation was expressed as change in mg-tension per cm of chart paper in resting tone (A). Relaxation was expressed in percentage, by considering VSL#3 mixture-induced maximal relaxation as 100% (B). The volume (μL) indicates the minimal value exerting maximal relaxation effects. Each crude extract was prepared from a bacterial suspension equivalent to a concentration of 3.5×10^5 CFUs/mL. Each point is the mean ± SE of 5 to 7 observations. Mean ± SE was given ($P < 0.05$).

successive additions of all crude extracts tested restored the basal tone of the tissue (data not shown). A further investigation was carried out with the crude extracts of VSL#3 *B. infantis* and *L. casei* strains, representative of *Bifidobacterium* and *Lactobacillus* genera. Colon relaxation patterns induced by these samples were identical to those obtained with the crude extracts of *Bifidobacterium* and *Lactobacillus* mixtures (data not shown).

Effects of *B. infantis* cell components on colon motility

To confirm that bacterial cytoplasmatic components are mainly responsible for colon relaxation, a more refined fractioning of the *B. infantis* cells was performed by separating cell wall fraction, membrane proteins, and cytoplasmatic fraction. A concentration-response curve was constructed for each of these bacterial portions (Figure 4). Indeed, the cytoplasmatic fraction showed a sustained relaxation of the colon segment equal to that demonstrated by the *B. infantis* crude extract. As expected, the cell wall fraction and the membrane proteins did not produce any significant effect.

The cytoplasmatic fraction of *B. infantis* was further refined in proteinaceous and non-proteinaceous components. The absence of protein in the non-proteinaceous fraction was verified by SDS PAGE analysis (Figure 5). As reported in Figure 6, the effect on colon relaxation was induced by the non-proteinaceous components, which showed a motility response similar to



<i>B. infantis</i>	μL	Relaxation %
Crude cell extract	1400	100
Cytoplasmatic fraction	1400	96 ± 3.5
Membrane fraction	1400	0
Cell wall fraction	1400	0

Figure 4 Relaxation induced by *B. infantis* crude extract, cytoplasmatic fraction, membrane fraction and cell wall fraction on Guinea-pig proximal colon. Relaxation was expressed as change in mg-tension per cm of chart paper in resting tone (A). Relaxation was expressed in percentage, by considering crude extract-induced maximal relaxation as 100% (B). The volume (μL) indicates the minimal value exerting maximal relaxation effects. Each crude extract was prepared from a bacterial suspension equivalent to a concentration of 3.5×10^5 CFUs/mL. Each point is the mean ± SE of 5 to 7 observations. Mean ± SE was given ($P < 0.05$).

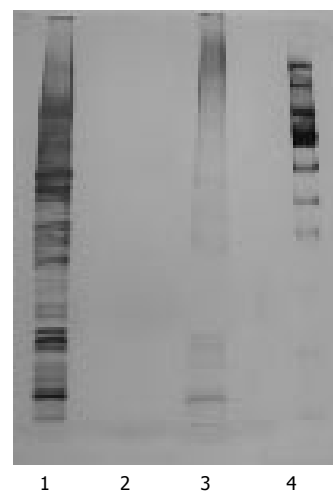


Figure 5 SDS PAGE electrophoresis of *B. infantis* cytoplasmatic fraction (lane 1), cytoplasmatic non-proteinaceous components (lane 2), cytoplasmatic fraction proteinase K digested (lane 3), molecular weight marker (lane 4).

that exerted by the cytoplasmatic fraction. The relaxation effect exerted by the non-proteinaceous components was confirmed by colon motility experiments carried out with a *B. infantis* cytoplasmatic fraction deprived of proteins by proteinase K digestion. This preparation, in which the enzymatic protein degradation was demonstrated by SDS PAGE analysis (Figure 5), evoked a significant colonic relaxation response similar to that obtained with the buffered non-proteinaceous preparation (Figure 6). To further characterize the *B. infantis* non-proteinaceous cytoplasmatic components, the possible involvement of bacterial genomic DNA fraction was tested. Addition to the organ bath of *B. infantis* DNA ranging from 0.5 μg to 10 μg did not modify the colon motility (data not shown).

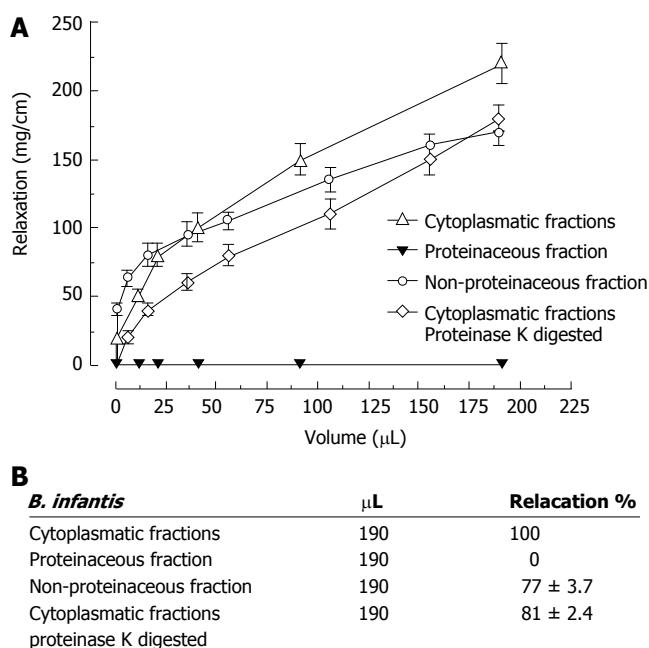


Figure 6 Relaxation induced by *B. infantis* cytoplasmic fraction, cytoplasmic proteinaceous components, cytoplasmic non-proteinaceous components and cytoplasmic fraction proteinase K digested on guinea-pig proximal colon. Relaxation was expressed as change in mg-tension per cm of chart paper in resting tone (A). Relaxation was expressed in percentage, by considering cytoplasmic fraction-induced maximal relaxation as 100% (B). The volume (μL) indicates the minimal value exerting maximal relaxation effects. Each crude extract was prepared from a bacterial suspension equivalent to a concentration of 3.5×10^9 CFUs/mL. Each point is the mean \pm SE of 5 to 7 observations.

DISCUSSION

The gut represents a complex and dynamic microbial ecosystem in which intestinal microflora has important and specific metabolic, trophic, and protective functions. Normal gut structure and function are the end-point of a complex set of interactions between the host and microorganisms colonizing the gut^[2]. Several studies have shown that probiotics restore mucosal integrity and regulate the immune response. However, the effects of probiotics on the enteric nervous system and the intestinal musculature have not been systematically studied. This study was designed to investigate the motility response evoked by the probiotic preparation VSL#3 on the ileum and proximal colon isolated from guinea-pigs.

The choice of VSL#3, a formulation with a high viable concentration of *Bifidobacterium*, *Lactobacillus* and *Streptococcus thermophilus*, was based on the reported efficacy in bowel diseases associated with changes in propulsive motility of the gut, including diarrhea-predominant irritable bowel syndrome^[18]. Furthermore, in clinical trials these exogenous probiotic bacteria have been shown to transiently colonize the human gut^[19, 21, 22].

The data from this study show that the motility of isolated ileum is not influenced by the addition of either entire cells or cell debris of the VSL#3 mixture, whereas its crude extracts induce a dose-dependent contraction. It is noteworthy that VSL#3 crude extracts, which have a pH of about 5, and crude extracts in which the pH was increased up to physiologic value of 7.4 showed

similar contractile responses. These results demonstrate that the ileum motility response is not related to the pH of the bacterial fraction and suggest that some bacterial metabolites could be involved in the contractions. Furthermore, as the presence of atropine (1 μmol/L) in the organ bath throughout the experiments did not determine a decrease of contraction induced by the addition of the VSL#3 crude extracts, the mechanism of action of the bacterial fractions does not involve muscarinic receptors.

Crude extracts of the different bacterial groups within the VSL#3 preparation provoke different motility response patterns. *Bifidobacterium* and *Streptococcus* strains did not change the basal tone of the ileum tissue, while *Lactobacillus* strains induced relevant ileum contractions. However, this contractile response is observed with volumes of cell extracts corresponding to *lactobacilli* concentration values of at least 10^7 CFU/mL, a value that is generally not detectable at ileum level of healthy humans^[27]. This result suggests that the contractile effect evoked in ileum by VSL#3 cell extracts will be of minor importance *in vivo* at ileum level, as the rapid transit of ileum content and the low bacterial titer do not allow it to reach sufficient bacterial lysis or metabolic secretion. Furthermore, it is noteworthy that the contractile response induced by *Lactobacillus* is lower than that promoted by crude extracts of intestinal *S. aboni* added at the same concentration.

Like the guinea-pig ileum, the motility of guinea-pig proximal colon segment is modified by crude extracts of the VSL#3 mixture, while entire bacterial cells and cell debris do not exert any effect. The crude extract of all the probiotic strains (*Bifidobacterium*, *Lactobacillus* and *S. thermophilus*) included in VSL#3 induced dose-dependent relaxation of the proximal colon tissue, whereas the crude extracts of the control strains did not influence colon motility. These results suggest that the ability to induce a colon relaxation response is specific for these probiotic bacteria. Furthermore, the proximal colon relaxation response induced by probiotic bacteria cell extracts is reversible as successive washings abolished the colon relaxation. The restoration of the basal tone of the proximal colon tissue after removing the stimuli by several washings suggests that colonic tissue is not damaged. Volumes of cell extracts from *Bifidobacterium*, *Lactobacillus* and *Streptococcus* inducing the relaxation response correspond to bacterial titer values that are in the range of the physiological concentrations measured in colon^[19].

Since *B. infantis* has been shown to colonize the human gut more efficiently than the other VSL#3 bacterial strains^[22], we used this strain to further investigate the components of probiotic bacteria involved in the guinea-pig colon relaxation. Interestingly, only the cytoplasmic fraction of the cell showed a pronounced colon relaxation activity. The bacterial cytoplasmic fraction may well have physiological implications, because its specific components reach the intestinal lumen as metabolites actively secreted by the viable bacteria colonizing the intestinal epithelium. In addition, the entire bacterial cytoplasm is released in the gut lumen following cell lysis. Further, subdivision of the bacterial cytoplasm allowed us to demonstrate that the

non-proteinaceous cytoplasmatic portion was responsible for the colon relaxation, whereas protein factors had no effect on proximal colon relaxation. This result has been confirmed by challenging the colon tissue with bacterial cytoplasmatic fractions digested with proteinase K.

Since the cytoplasmatic fraction deprived of the protein component contains significant amounts of genomic DNA, which in prokaryotic microorganisms is not confined to a nucleus, the proximal colon segment was tested with the bacterial genomic DNA. The DNA preparation extracted from *B. infantis* did not cause any changes in colonic motility, indicating that other unidentified non-proteinaceous factors may be involved.

In conclusion, this is the first study performed in isolated tissues that deals with the effects of probiotic bacteria on intestinal motility. Our results suggest that *Bifidobacterium*, *Lactobacillus* and *Streptococcus* strains in VSL#3 mediate proximal relaxation activity. This could be one of the possible mechanisms of action by which probiotic bacteria exert their effects in ameliorating diarrhea by reducing stool frequency and restoring a disturbed microbial balance. Based on these data, further studies to elucidate the implication of a large array of receptor families and subtypes known to affect gut function and to characterize the non-proteinaceous bacterial molecules involved in intestinal motility are warranted.

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Increased DNA binding activity of NF- κ B, STAT-3, SMAD3 and AP-1 in acutely damaged liver

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Gene regulation

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Abstract

AIM: To investigate the role of genes and kinetics of specific transcription factors in liver regeneration, and to analyze the gene expression and the activity of some molecules crucially involved in hepatic regeneration.

METHODS: USING gel-shift assay and RT-PCR, transcription factors, such as NF- κ B, STAT-3, SMAD3 and AP-1, and gene expression of inducible nitric oxide synthase (iNOS), hepatocyte growth factor (HGF) and c-met were analyzed in an animal model of chemically induced hepatectomy.

RESULTS: Gene expression of HGF and its receptor c-met peaked at 3 h and 24 h after acute CCl₄ intoxication. iNOS expression was only observed from 6 to 48 h. Transcriptional factor NF- κ B had an early activation at 30 min after acute liver damage. STAT-3 peaked 3 h post-intoxication, while AP-1 displayed a peak of activation at 48 h. SMAD3 showed a high activity at all analyzed times.

CONCLUSION: TNF- α and IL-6 play a central role in hepatic regeneration. These two molecules are responsible for triggering the cascade of events and switch-on of genes involved in cell proliferation, such as growth factors, kinases and cyclins which are direct participants of cell proliferation.

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Key words: Hepatic regeneration; Transcription factor;

INTRODUCTION

The liver is a unique organ with with ability to regulate its growth. This capacity can be altered at various different conditions like tissue dismissal and cell loss caused by chemical or viruses^[1]. Specific molecules are involved in the molecular events originated from these processes. One of these molecules is IL-6 which is an important factor for liver regeneration and repair after injury^[2]. IL-6-deficient mice fail in regenerating its hepatic gland presenting liver necrosis, functional failure, blunted DNA response in hepatocytes, absence of STAT-3 and NF- κ B activation, and selective dysfunction in AP-1, c-myc and cyclin D1 gene expression^[3]. IL-6 signals via STAT-3, and STAT-3 activation increase in an IL-6-dependent manner post-hepatectomy (PH) and post acute CCl₄ intoxication peaking at 2 h^[4,5] and returning to basal levels at 12 h^[6]. IL-6 is also an activator of AP-1 expression in liver regeneration^[3]. AP-1 and STAT-3 act in a synergistic fashion enhancing transcription^[7]. In our previous study IL-6 was strictly detected only at 24 h after acute CCl₄ intoxication. We could not detect IL-6 mRNA in rats intoxicated with turpentine, indicating the need of the presence of acute phase response for hepatocyte proliferation^[8]. On the other hand, TNF- α has also been shown as a major effector of signal pathways of liver regeneration^[9]. Several lines of evidence invoke the role of TNF- α in the regulation of IL-6 secretion through a previous induction of NF- κ B^[10]. Our previous results showed that induction of TNF- α gene expression takes place as early as 6 h, peaking at 48 h post-acute CCl₄ intoxication and this expression might induce IL-6 production^[8]. In correlation with our results, others have shown that TNF- α activates NF- κ B in many cells within 30 min after intra-peritoneal injection^[11]. TNF- α and IL-6 also induce iNOS transcription through NF- κ B activity^[12] which occurs principally in hepatocytes due to NO production,

and is detected in these cells just after partial hepatectomy and before cell proliferation^[13]. HGF, the major growth factor involved in hepatocyte proliferation, signals through its receptor c-met, a transmembrane tyrosine kinase protein product of the proto-oncogene with the same name^[14]. HGF is produced by hepatic stellate cells (HSCs) and acts on cultured hepatocytes in a paracrine manner as a potent mitogen^[14,15]. HGF production is induced in animals by partial hepatectomy and hepato-toxin^[16], detecting the mature form in significant levels^[17]. The results in animal models correlate with the elevated levels of serum HGF in patients with hepatic disorders^[18]. In this study, we aimed to elucidate the kinetic of activation of several transcription factors and molecular mechanisms involved in hepatic regeneration in an animal model. Furthermore, we shed light on how these transcription factors are involved in the resolution of this process. The early activation of NF- κ B, STAT-3 and AP-1 along with the expression of iNOS, HGF and c-met observed in this study suggested that induction of events like production of TNF- α , IL-6, HGF and some proteins are involved in cell proliferation. Knowledge obtained regarding activation of these transcription factors might enable us to propose new pharmacological strategies of treatment for induction of hepatic regeneration in some cases of cirrhosis.

MATERIALS AND METHODS

Animals

Forty male Wistar rats (Charles Rivers Inc., Boston, MA), weighting 200 g, were used in this study and housed according to the principles and procedures outlined by the National Institute of Health's Guide for the Care and Use of Laboratory Animals. For acute intoxication experiments, five rats for each time point were intragastrically administered a single dose of a mixture 1:1 (v/v) of CCl₄ (Merck Company, Darmstadt, Germany) and mineral oil (Sigma Chemical Company, St Louis MO, USA) at 5 mL/kg of body weight. Control animals were administrated a same volume of vehicle. Animals were sacrificed at 0.5, 1, 3, 6, 12, 24, and 48 h after CCl₄ intoxication. Livers were removed, immediately frozen in CO₂-acetone and stored at -70° until use.

Extraction and quantification of RNA

Isolation of total RNA from rat livers was carried out according to the modified method described by Chomczynski *et al*^[19]. Briefly, liver tissue was taken from three different lobes to obtain a representative sample and homogenized using a Polytron System (Brinkmann, Switzerland) in the presence of Trizol (Invitrogen). Chloroform was added, the aqueous phase was obtained and the RNA was precipitated from the aqueous phase by isopropanol at 4°C overnight. Quantity and intactness of RNA were routinely tested by determining absorbance (*A*) at 260/280 and ethidium bromide fluorescence of RNA electrophoresis on 10 g/L formaldehyde-containing agarose gels.

Analysis of iNOS, c-met and HGF gene expression

Expressions of iNOS, c-met and HGF gene were detected

using RT-PCR as previously described^[8]. We amplified the target genes iNOS, c-met, HGF and the constitutive gene HPRT in different reaction tubes. RNA from liver samples was isolated with Trizol and 2 g of total RNA was reverse transcribed into complementary DNA (cDNA) using 0.05 mol/L Tris-HCl (pH 8.3), 0.04 mol/L KCl, 0.007 mol/L MgCl₂ buffer containing 0.05 g/L random hexamers (Invitrogen), 0.001 mol/L dNTPs mix (Invitrogen), 50 U/L RNase inhibitor and 400 U of Moloney murine leukemia virus reverse transcriptase (M-MLV) (Invitrogen). Samples were incubated for 10 min at 25°C and then for 60 min at 37°C. Reverse transcriptase was further inactivated by heating the sample tubes at 95°C for 10 min. The cDNAs were used to perform PCR reaction according to the optimal amplification conditions for each gene. Amplification was performed in a PCR buffer of 0.05 mol/L Tris-HCl (pH 9.0) and 0.05 mol/L NaCl containing a mixture of 1×10^{-4} mol/L dNTPs and 1 unit of Taq DNA polymerase (Invitrogen). Amplification reactions were overlaid with light mineral oil and held at 94°C for "hot-start" PCR for 3 min and then run in an automated thermal cycler for different number of cycles and incubation temperatures according to each gene. Each PCR reaction was repeated at least in triplicate. Annealing temperature, number of cycles and primer sequence for each gene are shown in Table 1.

Nuclear extract isolation

Isolation of nuclear proteins was carried out according to the methods described by Andrews *et al*^[20], with a few modifications. Briefly, 1 g of liver from CCl₄-treated and controls rats was homogenized in 5×10^{-4} L of buffer A (0.01 mol/L Hepes-KOH (pH 7.9), 250 g/L glycerol, 0.420 mol/L NaCl, 0.0015 mol/L MgCl₂, 2×10^{-4} mol/L EDTA, 5×10^{-4} DTT, 2×10^{-4} PMSF) to disrupt extracellular matrix and cellular membranes. Homogenates were centrifuged at 1200 r/min for 10 s at 4°C. The pellet was resuspended in 2.5×10^{-4} L of buffer C (0.02 mol/L Hepes-KOH (pH 7.9), 250 g/L glycerol, 0.42 mol/L NaCl, 15×10^{-4} mol/L MgCl₂, 2×10^{-4} mol/L EDTA, 5×10^{-4} mol/L DTT, 2×10^{-4} mol/L PMSF), homogenized and incubated at 4°C for 20 min. Cellular debris was removed by centrifugation at 4°C for 2 min. Supernatant fraction containing DNA binding proteins was recollected and quantified as described by Bradford^[21]. Supernatant was stored at -70°C in aliquots until use.

Gel mobility shift assays

Electrophoretic mobility shift assay (EMSA) was performed as described elsewhere^[3]. Binding reactions were prepared using 2 g of nuclear extracts from either acutely damaged rat livers or from control animals. Additionally, 100 000 cpm of radioactive probe and 1.2 g of poly (DI-DC) as a non-specific DNA competitor were included in the binding reactions. Mixtures were incubated for 30 min at room temperature in binding buffer containing 0.01 mol/L Hepes (pH 7.5), 0.05 mol/L NaCl, 0.001 mol/L EDTA and 100 g/L glycerol. For competition assays, a 100-fold excess of unlabeled probe was added to the reactions concomitantly with the hot probe. For supershift experiments, 1 mL of antibody against NF- κ B,

Table 1 Oligonucleotide sequences used for PCR amplification

Gene	Sequence	Annealing temperature	Cycles	Size (bp)
HPRT	5' TCC CAG CGT CGT GAT TAG TG 3' 5' GGC TTT TCC ACT TTC GCT GA 3'	60°C	30	618
iNOS	5' TAG AGG AAC ATC TGG CCA GG 3' 5' TGG CCG ACC TGA TGT TGC CA 3'	58°C	25	255
c-MET	5' CAG TGA TGA TCT CAA TGG GCA AT 3' 5' AAT GCC CTC TTC CTA TGA CTT C 3'	60°C	28	725
HGF	5' AGC TCA GAA CCG ACC GGC TTG CAA CAG GAT 3' 5' TTA CCA ATG ATG CAA TTT CTA ATA TAG TCT 3'	60°C	27	618

Table 2 Oligonucleotide sequences used for EMSA

Transcription factor	Sequence
NF-κB	5' AGT TGA <u>GGG GAC TTT CCC</u> AGG C 3' 3' TCA ACT <u>CCC CTG AAA GGG</u> TCC G 5'
STAT-3	5' GAT CCT <u>TCT GGG AAT</u> TCC 3' 3' CTA GGA <u>AGA TCC TTA</u> AGG 5'
SMAD-3	5' TCG AGA GC <u>CAGA</u> CAA AAA GC <u>CAGA</u> CAT TTA GC <u>CAGA</u> CAC 3' 3' AGC TCT CG <u>GTCT</u> GTT TTT CG <u>GTCT</u> GTA AAT CG <u>GTCT</u> GTG 5'
AP-1	5' GAT CGA <u>TGA CTC AGA</u> GGA AAA 3' 3' CTA GCT <u>ACT GAG TCT</u> CCT TTT 5'

Bold and underlined letters denote specific consensus DNA-binding sequences.

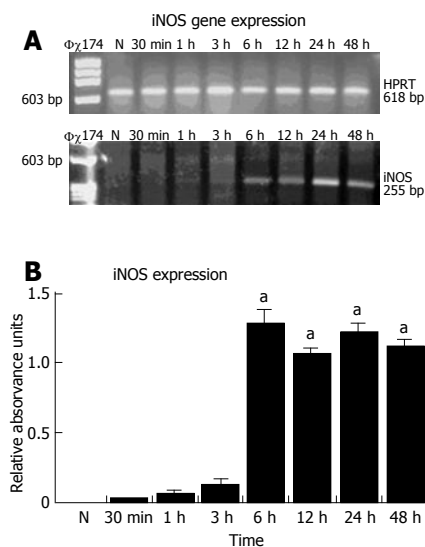


Figure 1 Semiquantitative RT-PCR analysis for iNOS expression. **A:** PCR products analyzed by agarose electrophoresis; **B:** normalized values of iNOS expression with respect to the housekeeping gene HPRT in all analyzed groups of three different PCR reactions performed.

STAT-3, AP-1 or SMAD3 (Santa Cruz Biotechnology) was incubated with the reaction mixture for 1 h at room temperature before regular incubation. The reactions were analyzed on 5% acrylamide non-denaturing gels in 0.5 × Tris-borate-EDTA buffer, dried and exposed. Intensity of each band, as the measure of DNA binding activity, was assessed by densitometric scanning Kodak ID 3.6 program. For gel retardation experiments, single-stranded oligonucleotides were obtained from Sigma and annealed

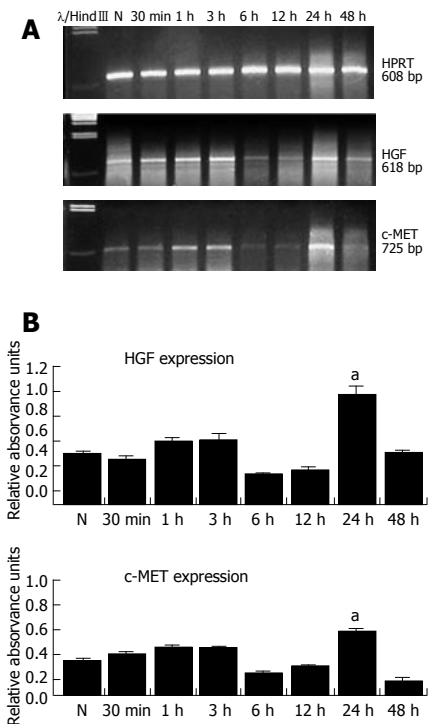


Figure 2 Semiquantitative RT-PCR analysis for HGF and c-met expressions. **A:** PCR products analyzed by agarose electrophoresis; **B:** Normalized values of HGF and c-met expression with respect to the housekeeping gene HPRT in all analyzed groups of three different PCR reactions performed.

in water. For annealing of complementary oligonucleotide pairs, 5 µg of each single-strand oligonucleotide was adjusted to a final volume of 5 × 10⁻⁵ L and placed on a heating blocker at 95°C for 5 min. Then the blocker was turned off and left to reach room temperature. Double-strand probe end labeling was performed using T4 polynucleotide kinase (Gibco) in the presence of (γ-32P) ATP. Each gel-shift experiment was performed in triplicate. Probe sequences for NF-κB, STAT-3, SMAD3 and AP-1 are shown in Table 2.

Statistical analysis

Results were expressed as mean ± SD. Student's *t* test was used to analyze the data. *P* < 0.05 was considered statistically significant.

RESULTS

After normalization against the housekeeping gene HPRT, iNOS expression was detected at 6 h after acute liver damage and continued up to 48 h, being statistically different at these times (*P* < 0.05). However, iNOS expression was not detected before 6 h (Figure 1).

The hepatocyte growth factor (HGF) and its cognate receptor c-met mRNAs after CCl₄ acute intoxication are presented in Figure 2. Two peaks of gene expression were observed in both genes: one at 3 h and another at 24 h where significant difference was seen only at 24 h (*P* < 0.05).

It has been shown that transcription factors are

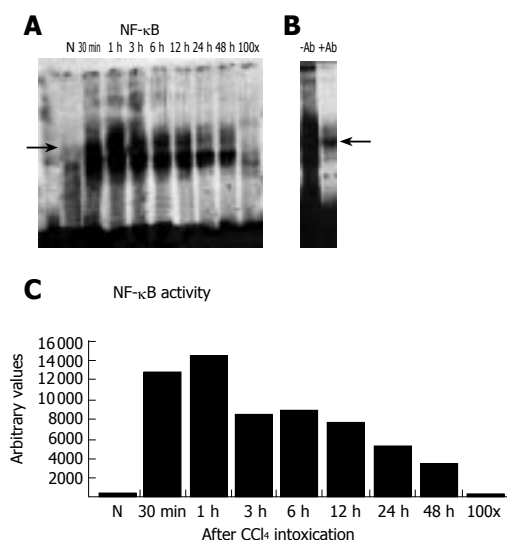


Figure 3 Binding activity of NF- κ B in acute liver damage. Wistar rats were intoxicated acutely with CCl₄ and then sacrificed at different time points. Nuclear proteins were extracted from the livers and Gel-shift assay was performed for NF- κ B transcription factor. **A**: Binding DNA activity of NF- κ B after CCl₄ intoxication; **B**: supershift assay using 1-h sample, 1 μ L of polyclonal antibody against p65 subunit was added and incubated for 1 h before usual EMSA assay; **C**: densitometric analysis of results of EMSA assay.

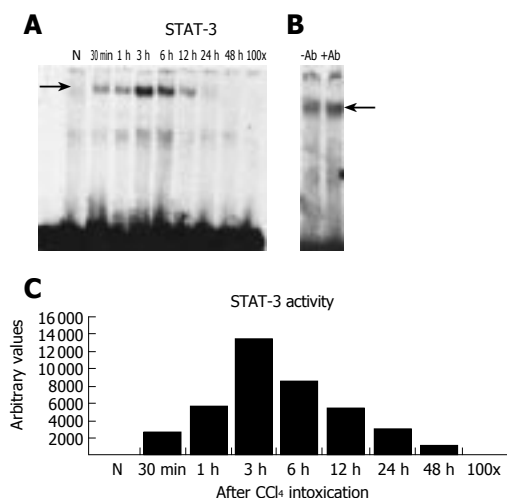


Figure 4 Binding activity of STAT-3 in acute liver damage. Wistar rats were intoxicated acutely with CCl₄ and then sacrificed at different time points. Nuclear proteins were extracted from the livers and Gel-shift assay was performed for STAT-3. **A**: Binding DNA activity of STAT-3 after CCl₄ intoxication; **B**: supershift assay similar using 6-h sample; **C**: densitometric analysis of results of EMSA assay.

activated during hepatic regeneration in different hepatectomy models^[9,22,23]. To determine the role and kinetic of activation of transcription factors critically involved in hepatic regeneration after acute CCl₄ injury, we performed gel-shift assays to evaluate the binding activity of NF- κ B, STAT-3, SMAD-3 and AP-1 on DNA probes containing consensus sequences. We performed a chronological analysis between 0.5 h and 48 h after liver damage, since it has been reported by others and us, that the inflammatory process has declined and cell proliferation has concluded by this time^[24]. Transcriptional

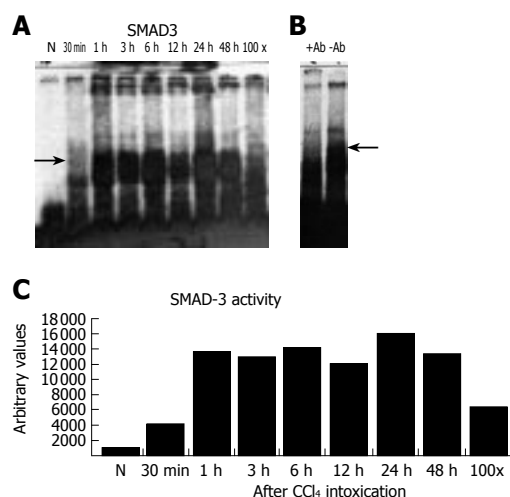


Figure 5 Binding activity of SMAD3 in acute liver damage. Gel-shift and supershift assays were performed to analyze SMAD3 DNA binding activity. **A**: Binding DNA activity of SMAD3 after CCl₄ intoxication; **B**: supershift assay using 24-h sample; **C**: densitometric analysis of results of EMSA assay.

factor NF- κ B presented strong DNA binding activity from 30 min after CCl₄-induced injury, peaking at 1 h and decreasing thereafter. Nevertheless, 48 h after CCl₄-induced injury, NF- κ B activity did not return to normal levels (Figure 3A). To confirm the specific binding activity of NF- κ B, we performed supershift assay using specific anti-NF- κ B antibodies. The use of a polyclonal antibody against the p65 subunit of NF- κ B decreased the binding of this transcription factor to the labeled probe, suggesting the binding of the antibody to the DNA binding site of the transcription factor (Figure 3B). Furthermore, almost complete elimination of the radiolabeled band, when a 100-fold cold DNA probe was used, confirmed the specificity of our results. These results were confirmed by densitometric analysis (Figure 3C).

With respect to STAT-3 activity, our results showed an increase at 30 min after intoxication, presenting the maximum activity at 3 h, and then decreasing and disappearing completely by 48 h (Figure 4A). We also made a supershift assay for this transcription factor obtaining a clear DNA binding reduction. The data suggest that, in our experimental conditions, this antibody preferentially binds to the DNA binding site on the transcriptional factor, thus hindering formation of STAT-3-DNA consensus site complex. This piece of data verifies that the results corresponded to this transcription factor (Figure 4B). These results were confirmed by densitometric analysis (Figure 4C).

On the other hand, SMAD3 showed a strong binding activity in this animal model at all analyzed times, as was confirmed by densitometric analysis at 1 h after CCl₄ intoxication until 48 h (Figures 5A and C). The specificity of the binding activity was analyzed by supershift assay (Figure 5B).

The transcription factor AP-1 showed a basal activity in normal animals. This activity increased as early as 30 min after CCl₄-induced injury and increased progressively showing the maximum peak in the last analyzed time in

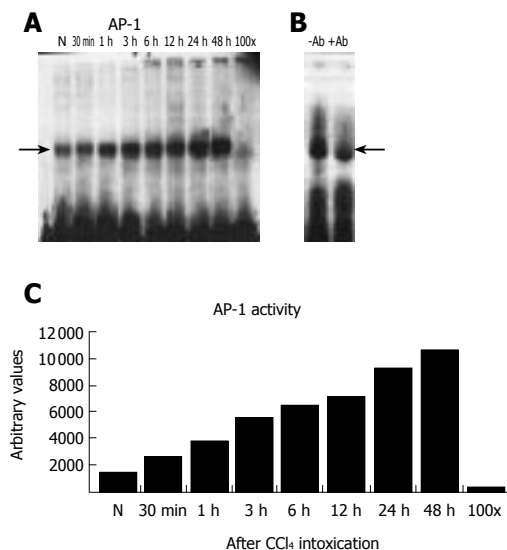


Figure 6 Binding activity of AP-1 in acute liver damage. EMSA and supershift assays, similar to Figures 3 and 4, were performed to analyze AP-1 activity in the same samples. **A:** Binding DNA activity of AP-1 after CCl₄ intoxication; **B:** supershift assay using 24-h sample; **C:** densitometric analysis of results of EMSA assay.

this study, 48 h after intoxication. Figures 6A-B clearly show the specificity of AP-1 DNA-binding activity when an excess of cold probe and specific anti-AP-1 antibody were used to override the binding of nuclear factor to the radiolabeled DNA-consensus sequence. These results were confirmed by densitometric analysis (Figure 6C).

DISCUSSION

The increase in DNA binding activity of the transcription factors NF- κ B, STAT-3 and AP-1 analyzed in this study indicates that hepatic regeneration process in response to CCl₄-induced acute liver damage requires the switch on and the switch off of many genes. These genes include cytokines, growth factors, kinases and cyclins which regulate cell cycle and induce hepatocyte proliferation. Our previous study demonstrated that expression of IL-6 is strongly associated with hepatic regeneration^[8]. We observed IL-6 gene expression in acute liver damage between 6 and 24 h, and disappeared thereafter. Other pro-inflammatory cytokines like IL-1 β and TNF- α also show strong expression around 24 h after liver damage^[24,25]. Because NF- κ B is required for TNF- α and iNOS production^[12], our results suggest that the strong inflammatory response present in CCl₄-intoxicated animals causes oxidative stress manifested as an increase in NF- κ B activity which induces iNOS expression. Although the maximum activity of NF- κ B was detected at 1 h post-CCl₄ intoxication, and iNOS expression was not observed before 6 h, suggesting that some hours are required for iNOS RNA to be detected. This inflammation is accompanied with damage and death followed by hepatocyte proliferation where TNF- α plays an important role^[26]. The activation of NF- κ B, STAT-3, SMAD3 and AP-1 allows their migration to the nucleus, where they can bind to their consensus sequence and induce the

expression of several genes involved in inflammation and cell proliferation^[27]. TNF- α promoter contains multiple binding sites for NF- κ B being a vital component for its expression and a ubiquitous oxidative stress-sensitive transcription factor^[28]. NF- κ B is found in almost all cell types, including hepatocytes and non-parenchymal cells^[29]. In absence of NF- κ B, TNF- α functions as an apoptotic agent in liver development. The NF- κ B inhibitor (I κ -B) degradation is enhanced by reactive oxygen species that can be generated by TNF and many other agents and cellular processes^[30].

Evidence indicates that blockade of NF- κ B in the regenerating liver by expression of NF- κ B super-repressor in an adenovirus vector leads to apoptosis after the cells have replicated their DNA^[31]. Similarly, introduction of I- κ B in an adenovirus vector after partial hepatectomy in mice results in increased liver injury and decreased hepatocyte cell proliferation.

On the other hand, transcription factor STAT-3 is also activated after partial hepatectomy but its activation is delayed compared to NF- κ B. STAT-3 becomes activated mainly by IL-6 type cytokines^[32]. Binding of IL-6 causes dimerization of the receptor, activation of tyrosine kinases which phosphorylate gp130 and create docking sites for STAT-3 binding. IL-6/STAT-3 signaling pathway is involved in cell proliferation through the induction of cyclins D1, D2, D3, A, cdc25A and concomitant down-regulation of cyclin-dependent kinase (cdk) inhibitors p21 and p27^[33,34]. p53, mdm2, p21, cyclins and cdk genes are also activated. STAT-3 activation observed in this study is in agreement with previous reports which demonstrated binding activity as early as 30 min using partial hepatectomy model to induce liver regeneration^[1,23]. SMAD3 have been found to be involved in hepatic stellate cell activation and collagen production after liver damage^[34], since high level of transcription factor SMAD3 could be detected even after 48 h of CCl₄ intoxication. These results suggest that liver damage caused by CCl₄ intoxication has not resolved at this time and some genes involved in the damage resolution activated by SMAD3 like TGF- α and collagen I are being expressed.

The role of AP-1 in the expression of molecule participants in cell proliferation, such as c-myc, D1 cyclin and cell growth factors, have been reported^[23]. The results obtained with AP-1 support, in fact, the role of AP-1 in hepatic regeneration. In this study, we observed a higher activity of AP-1 and also the maximum hepatocyte proliferation between 24 h and 48 h after CCl₄-induced liver damage, which are in agreement with previous studies^[35,36]. The initiation step called “priming step” appears to be mediated by TNF- α and IL-6 and their downstream pathways involving activation of NF- κ B, STAT-3 and AP-1^[1,37]. Activation of these transcription factors leads the progression to G1 phase of the cell cycle^[33]. “Priming” of hepatocytes induces them to respond to extra- and intra-hepatic growth factors, such as epidermal growth factor (EGF), transforming growth factor- β (TGF- β) and HGF^[1]. In this study, we found HGF gene expression and its receptor c-met peaking at 1 and 24 h after the liver damage. These results allow to us think that HGF expression could be induced by two

different pathways involving two different molecules, first TNF- α and then IL-6. Since HGF strongly stimulates DNA synthesis in damaged hepatocytes, an increase in HGF and its receptor mRNA expression suggests that liver regeneration is taking place^[15,17].

TNF- α signals through two distinct receptors: TNFR-1 and TNFR-2^[38]. Mice lacking functional TNFR-2 show completely normal DNA replication after hepatectomy and CCl₄ treatment. In contrast, lack of signaling through TNFR-1 greatly inhibits DNA replication after partial hepatectomy and cause significant mortality 24-40 h after the operation^[39]. In TNFR-1-knockout mice, activation of NF- κ B and STAT-3 is inhibited and AP-1 activation is decreased. The signal transduction pathway starting from TNF- α required for liver regeneration involves TNFR-1 with NF- κ B activation. The sequence of events proposed for liver regeneration after CCl₄-intoxicated acute liver damage is similar to that observed with different animal models like partial hepatectomy. However, the time of activation seems to be more delayed. The sequence of these events is as follows: TNF- α binds to TNFR-1 and induces activation of NF- κ B, NF- κ B binds to IL-6 promoter and the protein is produced. IL-6 activates STAT-3, which in turn activates AP-1. AP-1 participates in expression of the genes involved in hepatocyte proliferation, such as D1 cyclin, c-myc and kinases^[7,9].

In conclusion, TNF- α and IL-6 are responsible for triggering the cascade of events and switch-on of genes involved in cell proliferation, such as growth factors, kinases and cyclins which are direct participants of cell proliferation.

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CLINICAL RESEARCH

Reduced expression of Ca^{2+} -regulating proteins in the upper gastrointestinal tract of patients with achalasia

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be of lesser importance for regulation of SERCA than in heart. Lower expression of Ca^{2+} storage proteins (CSQ and CRT) might contribute to increased lower esophageal sphincter pressure in achalasia, possibly by increasing free intracellular Ca^{2+} .

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Key words: Esophageal and gastric motility; Esophagus; Calsequestrin; Calreticulin

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Abstract

AIM: To compare expression of Ca^{2+} -regulating proteins in upper gastrointestinal (GI) tract of achalasia patients and healthy volunteers and to elucidate their role in achalasia.

METHODS: Sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) isoforms 2a and 2b, phospholamban (PLB), calsequestrin (CSQ), and calreticulin (CRT) were assessed by quantitative Western blotting in esophagus and heart of rats, rabbits, and humans. Furthermore, expression profiles of these proteins in biopsies of lower esophageal sphincter and esophagus from patients with achalasia and healthy volunteers were analyzed.

RESULTS: SERCA 2a protein expression was much higher in human heart (cardiac ventricle) compared to esophagus. However, SERCA 2b was expressed predominantly in the esophagus. The highest CRT expression was noted in the human esophagus, while PLB, although highly expressed in the heart, was below our detection limit in upper GI tissue. Compared to healthy controls, CSQ and CRT expression in lower esophageal sphincter and distal esophageal body were significantly reduced in patients with achalasia ($P < 0.05$).

CONCLUSION: PLB in the human esophagus might

INTRODUCTION

Intracellular Ca^{2+} regulates contractility in striated, smooth, and heart muscle. In the heart, expression of the most important proteins for intracellular Ca^{2+} homeostasis has been studied extensively. β -adrenoceptor agonists elevate Ca^{2+} by activation of cAMP-dependent protein kinase (PKA) and subsequent phosphorylation of regulatory proteins. The predominant protein phosphorylated in cardiac sarcoplasmic reticulum (SR) by PKA is phospholamban (PLB). The best characterized function of phospholamban is the regulation of the activity of the SR or endoplasmic reticulum (ER) Ca^{2+} -pump (SERCA). In isolated guinea-pig hearts, β -adrenergic stimulation leads to phosphorylation of PLB and, at the same time, Ca^{2+} -uptake into the SR is increased^[1]. PLB in its dephosphorylated state lowers the Ca^{2+} affinity of SERCA, while phosphorylation of PLB reduces the affinity of SERCA for Ca^{2+} . Therefore, PLB phosphorylation increases the rate of Ca^{2+} -transport into the SR. As a consequence, the rate of cardiac relaxation as well as contraction increase^[2,3]. SERCA itself is coded by three genes in mammals: SERCA 1, 2, and 3^[4]. SERCA 1 usually is expressed in fast-twitch skeletal muscle and SERCA 3 in non-muscle cells. For SERCA 2, two splice variants are known: SERCA 2a and SERCA 2b, the latter containing 49 additional amino acids at the carboxyterminal end^[5]. In the heart SERCA 2a

is the predominant isoform, whereas SERCA 2b is found mainly in smooth muscle, but smaller amounts have been recognized also in other tissues like heart^[6].

Intracellular rapidly exchanging Ca^{2+} stores provide the possibility for release, uptake, and storage of Ca^{2+} transported into SR by SERCA. Ca^{2+} -binding proteins with high capacity and low affinity for Ca^{2+} -binding permit rapid Ca^{2+} release. At least two families of these Ca^{2+} -binding proteins have been described, namely calsequestrin (CSQ) and calreticulin (CRT). CSQ is found in skeletal muscle and heart, while CRT is more widely distributed^[7,8]. CSQ is coded by two genes, the skeletal muscle isoform (CSQ1) coding for a 62-ku protein, and the cardiac isoform (CSQ2) coding for a 51-ku protein^[9,10]. Differences within the amino acid sequences might be functionally relevant, because the deduced amino acid sequence of cardiac calsequestrin is consistent with its ability to bind larger amounts of Ca^{2+} ^[10]. In contrast, CRT is coded by one gene and splice variants have not yet been reported^[11].

In heart, regulation of SERCA2a activity by expression and phosphorylation of PLB is well established, and disturbances of this system might be involved in pathophysiological events leading to heart failure^[3,12,13]. Indeed, targeted over-expression of CSQ or CRT in the heart of transgenic mice led to cardiac hypertrophy, dilated cardiomyopathy, and heart failure^[14-16]. However, expression, function, and pathophysiological role of Ca^{2+} -processing proteins in the human gastrointestinal (GI) tract and their alterations in diseased states have not been examined before.

There are few diseases of the GI tract where altered Ca^{2+} homeostasis might be involved. One of the possible entities is achalasia. Achalasia is a rare motility disorder characterized by increased pressure of the lower esophageal sphincter (LES) and simultaneous contractions of the esophageal body (EB) leading to impaired swallowing. There is evidence that gastric motility is also impaired in achalasia^[17,18]. Common treatments of achalasia are invasive procedures like pneumatic dilatation and laparoscopic myotomy. For medical therapy, smooth muscle relaxants as nitrates and Ca^{2+} -channel blockers of the dihydropyridine-type are effective in the treatment of achalasia^[19]. Hence, we tested the hypothesis that the expression of SR-proteins involved in Ca^{2+} handling in the GI tract is altered in achalasia in humans.

MATERIALS AND METHODS

Animals

Male Wistar rats (body weight, 150-250 g) and rabbits which had fasted overnight were sacrificed. Organs (esophagus, heart) were prepared and immediately frozen in liquid nitrogen.

Human cardiac tissue

Samples were taken from left ventricles of non-failing hearts which were obtained from prospective organ donors whose hearts could not be used. The study was performed in accordance with the guidelines from the Local Ethics Committee.

Patients

Manometrically proven 9 patients with achalasia (age, 19-52 years), and 6 healthy controls (age, 22-49 years) participated in the study. None of the control group had a history of GI disease or complained of any symptom of a GI disease. All patients underwent gastroduodenoscopy and ¹³C-urea breath test to exclude gastroduodenal disease or infection with *H. pylori*. During endoscopy, biopsies of esophagus and LES were taken. Biopsies were immediately frozen in liquid nitrogen. The study had been approved by the Local Ethics Committee, and all patients gave written informed consent.

Esophageal manometry

Patients were fasted overnight before manometric examination was performed using a low-compliance, water-perfused system. The Arndorfer catheter-with four side-holes oriented radially, 0.5 cm apart, located distally for LES (lower esophageal sphincter) examination and four more orifices located every 5 cm above the distal ones thus allowing for the assessment of the motility pattern in the middle and distal part of the EB (esophageal body)-was attached to force transducers. The recorded signals were amplified (Polygraph VIII, Synectics, Stockholm, Sweden) and stored for further analysis with a specially designed software (Gastrosoft, Irving, Texas, USA). The motility patterns of distal esophagus were examined with (wet swallows) and without (dry swallows) swallowing of 5 mL of water bolus and expressed as mean values of the amplitude (mm Hg), duration (s) and propagation (cm/s) as calculated for five wet and five dry swallows. The LES resting tone was assessed by pull-through technique and the mean value, expressed in mm Hg above the mean intragastric pressure, was calculated from three consecutive measurements. The LES relaxation was measured in response to 5 mL of water bolus, while the distal catheter orifices were located in the LES high-pressure zone and results were documented as complete or incomplete relaxation of the LES.

SDS-PAGE and Western blot analysis

Gel electrophoresis was performed according to the method described by Laemmli^[20]. Briefly, samples were homogenized in 10 mmol NaHCO_3 /50 g/L SDS using a microdismembrator (Braun, Melsungen, Germany) and the protein content of homogenates was measured according to Lowry *et al.*^[21]. After SDS polyacrylamide gel electrophoresis, the separated proteins were transferred onto nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany) as described previously^[22]. For immunostaining, the following primary antibodies were used: monoclonal anti-PLB (2D12), monoclonal anti-SERCA 2a (2A7-A1), polyclonal anti-CSQ (all kindly provided by L.R. Jones, Krannert Institute of Cardiology, Indianapolis, USA), polyclonal anti-SERCA 2b (kindly provided by L. Raeymaekers and Wuytack, University of Leiden, Belgium), and polyclonal anti-calreticulin (Alexis, Grünberg, Germany). Thereafter, bound primary antibodies were detected with [¹²⁵I]-labeled protein A (ICN, Meckenheim, Germany). Visualization and

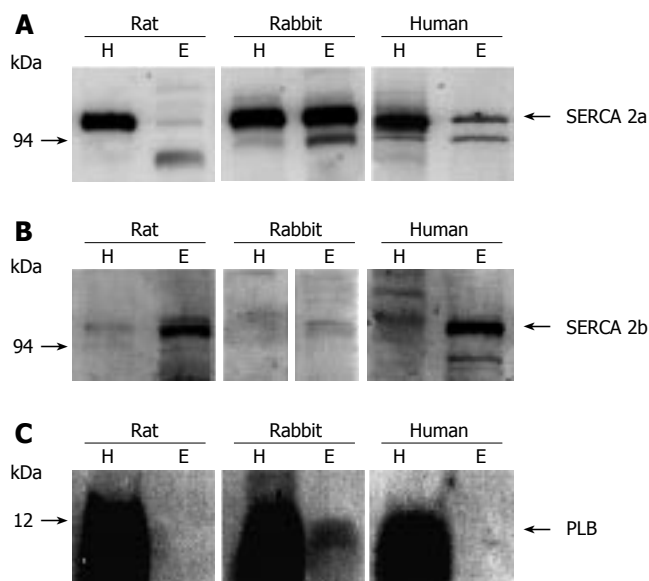


Figure 1 Protein expression of SERCA 2a (A), 2b (B), and PLB (C) in esophageal and ventricular homogenates of rat, rabbit, and human. Protein loading for esophagus (100 μ g) was 5 times higher than for heart (20 μ g). For documentation of PLB, sensitivity of the PhosphorImager was optimized for detection of very weak signals. Therefore, signals in heart samples are overexposed. H: heart; E: esophagus.

quantification of protein bands were performed with a PhosphorImager system (Molecular Dynamics, Krefeld, Germany). Differences in protein loading were corrected by densitometric quantification of Ponceau-stained membranes.

Statistical analysis

Results were presented as mean \pm SE. The significance of differences between means was evaluated using Mann-Whitney test with a confidence value at $P < 0.05$.

RESULTS

First, we studied the expression of Ca^{2+} -regulatory proteins in the upper GI tract. For comparison, we used cardiac tissues where the distribution of Ca^{2+} -regulatory proteins has been established before^[23,24]. Finally, we were interested in their putative pathophysiological role in man. Of note, to our knowledge, expression of these proteins has not been studied before in the human GI tract. However, as more data on expression are available for animal tissues than for human tissues, we studied, where possible, human and animal tissues in parallel. First, we analyzed the expression of SERCA in homogenates from various tissues. Figure 1 depicts protein expression of SERCA 2a and 2b in the esophagus and heart of different species (human, rat, and rabbit). Although, only 20 μ g of ventricular protein was loaded compared to 100 μ g for esophagus, it is obvious that SERCA 2a protein expression was highest in the cardiac ventricle in all three species analyzed (Figure 1A). In rabbit esophagus, the SERCA 2a signal was quite pronounced, whereas in human and rat esophageal expression of SERCA 2a was hardly detectable (Figure 1A). Although, primary sequences of SERCA 2a

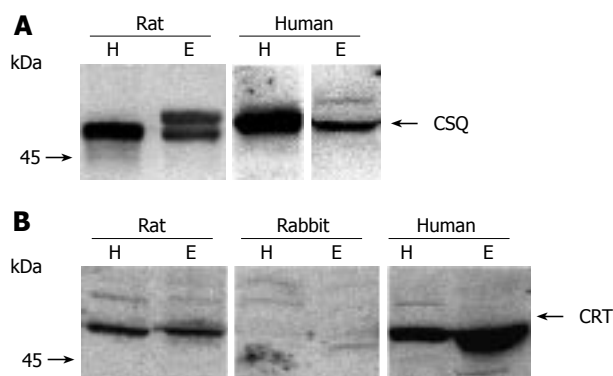


Figure 2 Protein expression of CSQ (A) and CRT (B) in esophageal and ventricular homogenates of rat, rabbit, and human. Protein loading was 50 μ g/lane for CSQ and 100 μ g/lane for CRT. H: heart; E: esophagus.

are somewhat different between rat, rabbit, and human, these expressional differences are valid, as comparisons only were done within one species^[4]. However, the SERCA 2a protein sequence expressed in esophagus and heart of a given species is identical. In rat and human, where SERCA 2a expression was barely detectable in 100 μ g of esophagus protein, a strong signal was visible in 20 μ g of cardiac proteins (Figure 1A). This strongly argues for tissue differences in expression between esophagus and heart at least in rat and, more importantly, in human. In contrast, SERCA 2b expression could be found predominantly in rat and human esophagus, while rabbit esophagus did contain only very low levels of SERCA 2b (Figure 1B). Only a very weak signal for SERCA 2b was detectable in heart, as expected (Figure 1B). For subsequent quantification, it was important to establish the linearity of protein detection by Western blotting. Detection of SERCA 2b in human left ventricle and esophagus as well as in rat samples was linear over a range of 50 to 200 μ g of protein loaded per lane (data not shown).

Next, we compared PLB expression in rat, rabbit, and human samples (Figure 1C). Strong signals could be detected in all homogenates of cardiac ventricles, while in rat and human esophagus, expression of PLB was below our detection limit (defined as 5% over background). Only in rabbit esophagus, a weak PLB signal was detectable (Figure 1C).

Both CSQ isoforms were expressed in esophagus of human and rat (Figure 2A). In the heart, only the low molecular weight isoform was present (Figure 2A). The high molecular weight band was identified as the skeletal muscle isoform and the lower band as the cardiac isoform of CSQ, based on the literature^[7].

We studied protein expression of CRT in all tissues. Expression of CRT in rat was nearly the same in esophagus and cardiac ventricle (Figure 2B). In humans, CRT expression was lower in heart than in esophagus (Figure 2B). In rabbit, CRT signal was much weaker in both tissues compared to human and rat. Conceivably this was a problem of the specificity of the antibody we used for detection of CRT.

After determination of protein expression of SERCA, PLB, CSQ, and CRT in different species, we posed the

Table 1 Ratios of CRT/CSQ and CRT/SERCA 2b in esophageal body (EB) and lower esophageal sphincter (LES) and LES pressure of achalasia patients and healthy controls (mean \pm SE)

	EB		LES	
	Control (n = 6)	Achalasia (n = 9)	Control (n = 6)	Achalasia (n = 9)
Pressure (mm Hg)	ND	ND	31.2 \pm 2.9	49.2 \pm 3.8 ^a
CRT/CSQ	0.63 \pm 0.08	0.61 \pm 0.04	0.88 \pm 0.10	0.78 \pm 0.08
CRT/SERCA 2b	1.07 \pm 0.20	0.77 \pm 0.10	1.47 \pm 0.09	1.07 \pm 0.08 ^a

CRT: Calreticulin; CSQ: Calsequestrin; SERCA: Sarcoplasmic reticulum Ca²⁺ ATPase; ND: Not done. ^aP < 0.05 vs controls.

question, whether expression of these proteins might be altered during GI disease. Ca²⁺ plays an important role in GI motility. Hence, we compared SERCA 2b, CSQ, and CRT expression in healthy controls and patients with achalasia (LES pressure was elevated by 58%, Table 1). In biopsies from LES and EB, SERCA 2b expression was not obviously different between controls and patients (Figure 3). However, CSQ expression in LES and EB was found to be significantly reduced in patients with achalasia (LES reduced by 19%, and EB by 22%) (Figure 3). CRT expression was reduced by 26% in LES as well as in EB of patients with achalasia (Figure 3). CRT/CSQ ratio was not markedly different between both groups, while CRT/SERCA 2b ratio was significantly reduced by 27% in LES of achalasia patients (Table 1).

DISCUSSION

For phasic smooth muscles, it was shown that SR Ca²⁺ cycling can play a major role in modulating mechanical activity^[25]. As achalasia, a human GI motility disorder, can be treated with drugs modulating intracellular Ca²⁺, one can speculate that impaired Ca²⁺-homeostasis might be involved in development or deterioration of this disease. Therefore, aim of this study was to compare protein expression of Ca²⁺-regulating proteins in upper GI tract from different species and moreover, to ascertain if they could play a role in human diseases of the upper GI tract. In rats, as well as in humans, SERCA 2a was predominantly expressed in cardiac tissue, while expression was hardly detectable in esophageal tissue. However, rabbit esophagus contained high amounts of SERCA 2a, while SERCA 2b was not detectable in both, cardiac and esophageal, homogenates of this species. Our data concerning the relative expression of SERCA 2a and SERCA 2b in rat cardiac and esophageal homogenates are in accordance with observations by Wu *et al.*^[26] who reported a similar ratio of SERCA 2a/2b expression in rat heart (30:1) and esophagus (1:4) at the mRNA level. Similar distribution of SERCA 2a and 2b RNAs in rat stomach and heart have been described^[27]. However, esophagus was not analyzed in this study. Another study, using the sensitive nuclease protection assay, determined a SERCA 2a/2b mRNA ratio of 20:1 in rabbit cardiac tissue, whereas in most smooth muscle and non-muscle tissues SERCA 2b was expressed predominantly^[28]. Surprisingly, in rabbit esophagus, SERCA 2a accounted for 80% of SERCA 2 content^[28].

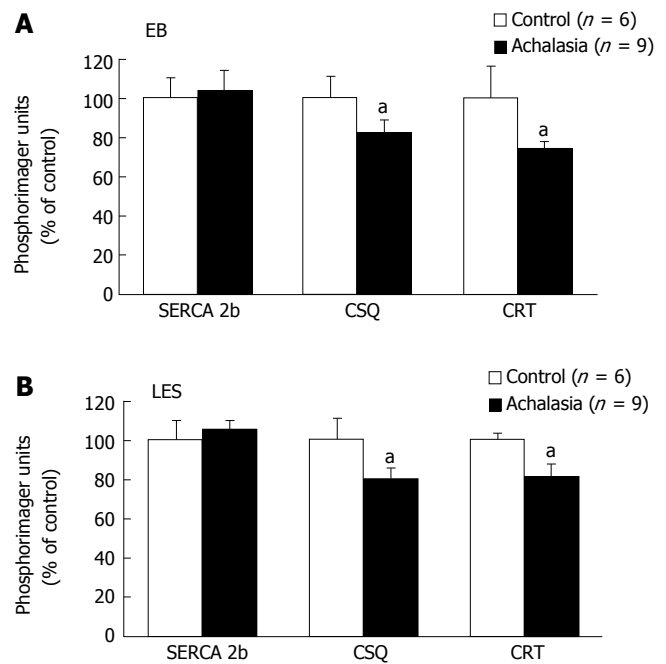


Figure 3 Protein expression of SERCA 2b, CSQ, and CRT in biopsies of esophageal body (EB, **A**) and lower esophageal sphincter (LES, **B**) from patients with achalasia or healthy controls. Protein expression was quantified using [¹²⁵I]-labeled protein A for detection of primary antibodies. Radioactive bands were visualized and quantified using a PhosphorImager. Data are presented as mean \pm SE of *n* patients. ^aP < 0.05 vs control.

These data are in accordance with our observation that in rabbit esophagus SERCA 2a expression was much more pronounced than in esophagus of rats and humans (compared to heart of the same species). Correspondingly, the SERCA 2b signal was significantly weaker in rabbit than in rat and human esophagus. Although, species specificity of the antibody (the alternatively spliced 50 amino acid carboxy terminal tail exhibits 10 differences between rabbit and man, 3 between rat and human and 10 between rabbit and human, most of these changes being conservative replacements^[28]) might contribute to this phenomenon, it is conceivable that the weaker expression of SERCA 2b might be due to a lesser importance of this isoform in rabbit esophagus. In summary, there are differences in the relative expression of SERCA 2a and 2b. Hence, splicing of SERCA 2 exhibits distinct tissue and species differences. Similar results at the protein level had been published elsewhere^[29]. But esophageal tissue was not analyzed so far.

In our study, expression of PLB was abundant in cardiac tissue of all species analyzed. A weak PLB signal could be obtained in rabbit esophagus. In the esophagus of rats and humans, PLB was below the detection limit. Only sparse data on PLB expression in the GI tract are available. Varying amounts of PLB mRNA and protein with a strong signal in gastric smooth muscle of pigs, but no signal in aorta were noted^[30]. In canine smooth muscle cells of ileum, PLB was detected by immunogold microscopy, but only ileum, not esophagus, was analyzed^[31]. Another group detected PLB in pig stomach, rat aorta and dog aorta, but not in pig aorta. They concluded from their experiments that PLB might exist in many, but not all muscle tissues^[32].

However, in our experiments, we failed to detect PLB in human and rat esophagus. This has not been studied and reported before. Our observation might be due to the fact that PLB is only of minor functional importance for SERCA regulation in these tissues.

May these discrepancies have functional consequences? Some studies have addressed the question of how SERCA 2a and 2b interact with PLB in different muscle tissues. The effect of cAMP- and cGMP-dependent protein kinases on Ca^{2+} uptake of ER vesicles is smaller in smooth muscle than in cardiac tissue^[33]. Using transfection experiments with COS-1 cells, a slower turnover rate for SERCA 2b was found and a higher apparent affinity for Ca^{2+} was reported for SERCA 2b compared to SERCA 2a. Ca^{2+} -affinity was decreased in both cases by co-expression with PLB^[5,34]. John *et al*^[35] reported similar functional differences of SERCA 2a and 2b isoforms. Additionally, they described the ability of CRT to modulate SERCA 2b-, but not SERCA 2a-activity, most likely by a binding to the additional eleventh transmembrane segment and luminal carboxy terminus of SERCA 2b. Since PLB expression in human and rat esophagus was below the detection limit, in contrast to cardiac PLB expression, esophageal PLB (at least in rats and humans) seems to be of lesser importance for regulation of SERCA than in heart. It is tempting to speculate that regulators other than PLB, possibly CRT, might control SERCA 2b and Ca^{2+} -handling in these tissues. In agreement with this speculation, we detected PLB expression only in rabbit esophagus, where a high amount of SERCA 2a, but no CRT was present, while CRT was detectable in rat and human esophagus, where SERCA 2b, but not PLB, was expressed.

In accordance with the literature, we found two isoforms of CSQ in rat, a low and high molecular weight isoform. In the heart exclusively the low molecular form was expressed, while smooth muscle tissue contained both isoforms^[7]. Here we have demonstrated that human esophagus also contains both isoforms of CSQ. To the best of our knowledge, CSQ protein expression in human upper GI tract has not been described before.

CRT protein expression was found in esophagus of rat and man and, to lesser extent, in cardiac tissue of these species, while it was missing in rabbit tissue. CRT expression, although being detectable in high amounts in various tissues, has not been analyzed in human esophagus. The expression patterns, presented here, encourage the speculation that CRT might be involved in the regulation of esophageal SERCA 2b.

Finally, we found decreased expression of CSQ and CRT in LES and EB of patients with achalasia. Although, antagonists of L-type Ca^{2+} -channels like nifedipine have proven effective in treatment of achalasia, studies on free intracellular Ca^{2+} levels in achalasia have not been published. It might be speculated that decreased CRT and CSQ levels might lead to increased intracellular free Ca^{2+} and therefore to elevated LES pressure. Interestingly, over-expression of CSQ decreases the free Ca^{2+} in the cytosol of cardiac myocytes^[15]. This finding may explain why contractility of CSQ-over-expressing cardiac cells was diminished compared to wild-type cells. In contrast, one can speculate that reduced expression of CSQ might

be accompanied by increased free cytosolic Ca^{2+} followed by enhanced contractility. If this mechanism comes true for smooth muscle cells, reduced expression of CSQ might lead to elevated free Ca^{2+} levels and hence increased tension and therefore to achalasia in patients. Reduced CRT expression might contribute to achalasia by similar mechanisms like PLB. For instance, in PLB-knockout mice, cardiac contractility was enhanced, because loss of PLB relieved any inhibition of SERCA function by PLB: Ca^{2+} is more effectively removed from the cytosol and more Ca^{2+} can be released during the systole leading to enhanced force generation in these hearts^[36]. A similar mechanism might hold true for CRT in esophagus: lower levels of CRT should also loosen its inhibitory modulation of SERCA function in the esophageal cells and more tension might be generated.

In conclusion, our results provide new insights to understand the mechanisms leading to achalasia. Further studies will be necessary to elucidate the role of impaired Ca^{2+} regulation for development of diseases like achalasia. Some questions which have to be answered are why the Ca^{2+} -regulatory proteins CSQ and CRT are down-regulated, and does down-regulation occur before or after manifestation of the disease.

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CLINICAL RESEARCH

Postprandial transduodenal bolus transport is regulated by complex peristaltic sequence

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Abstract

AIM: To study the relationship between the patterns of postprandial peristalsis and transduodenal bolus transport in healthy subjects.

METHODS: Synchronous recording of chyme transport and peristaltic activity was performed during the fasting state and after administration of a test meal using a special catheter device with cascade configuration of impedance electrodes and solid-state pressure transducers. The catheter was placed into the duodenum, where the first channel was located in the first part of the duodenum and the last channel at the duodenojejunal junction. After identification of previously defined chyme transport patterns the associated peristaltic patterns were analyzed.

RESULTS: The interdigestive phase 3 complex was reliably recorded with both techniques. Of 497 analyzed impedance bolus transport events, 110 (22%) were short-spanned propulsive, 307 (62%) long-spanned propulsive, 70 (14%) complex propulsive, and 10 (2%) retrograde transport. Short-spanned chyme transports were predominantly associated with stationary or propagated contractions propagated over short distance. Long-spanned and complex chyme transports were predominantly associated with propulsive peristaltic patterns, which were frequently complex and comprised multiple contractions. Propagated double wave contraction, propagated contraction with a clustered contraction, and propagated cluster of contractions have been identified to be an integrated part of a peristaltic sequence in human duodenum.

CONCLUSION: Combined impedancometry and manometry improves the analysis of the peristaltic patterns that are associated with postprandial transduodenal chyme

transport. Postprandial transduodenal bolus transport is regulated by propulsive peristaltic patterns, which are frequently complex but well organized. This finding should be taken into consideration in the analysis of intestinal motility studies.

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Key words: Transduodenal bolus transport; Organization of duodenal peristalsis; Combined impedance manometry

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INTRODUCTION

The spatial and temporal organization of gastrointestinal contraction waves seems to be a more important determinant of the flow of luminal contents than their number and amplitude^[1,2]. Therefore, significant advances in understanding intestinal motility disorders could be made by analysis of bolus movement together with peristaltic activity within the gastrointestinal lumen. With manometry alone small intestinal motility patterns can be obtained^[3]. However, up to date there are limited observations on the relationship between intestinal chyme transport and peristaltic activity in humans because fluoroscopic studies are limited due to radiation exposure.

Multichannel impedancometry is a newly developed technique to study chyme transport^[4]. We performed human studies and demonstrated that multichannel impedancometry is a reliable technique to obtain detailed information about spatial and temporal chyme movements, both in the human esophagus and duodenum^[5-9]. Simultaneous impedancometry and pH-monitoring have also been used for characterisation of patterns of gastroesophageal reflux^[10,11].

In previous studies we have characterized postprandial duodenal chyme transport patterns^[5,6]. Recently, we developed the technique of combined impedancometry and manometry (CIM)^[12] and applied it for motility testing in healthy subjects and reflux patients^[13-15]. In the present study we used this approach in order to systematically

study the relationship between intestinal chyme transport patterns and peristaltic patterns and to obtain detailed information about peristaltic mechanisms regulating postprandial transduodenal bolus transport.

MATERIALS AND METHODS

Subjects

Ten subjects (7 males and 3 females, mean age 34 years, range 26-36 years) were studied after written informed consent. All healthy volunteers were recruited from the medical staff. They took no medication and had no history of gastrointestinal disease. The study protocol was approved by the local ethical committee of Aachen University.

Methods

Combined impedance and pressure recording: A custom-made combined catheter consisting of 11 impedance segments (each 2 cm long) and 4 semiconductor pressure transducers was used (prototype developed by Dr. Nguyen RWTH-Aachen, Ref. 13). The pressure transducers were located between the impedance channels 1-2, 4-5, 7-8 and 10-11 (intertransducer distance 6 cm) (Figure 1). The cascade configuration of the impedance electrodes allows continuous monitoring of chyme movement and is particularly suitable for analysis of chyme transport patterns^[5,6].

Study protocol: After an overnight fast from 10 pm, the catheter was placed transnasally into the duodenum under fluoroscopic control. All channels were placed in the duodenum. The proximal end of the catheter was located in the first part of the duodenum and the last channel at the duodenojejunal junction (Figure 1). This catheter position yielded information about bolus transport along the whole duodenum. The final position was confirmed by fluoroscopy at the beginning of the studies.

Synchronous recording of chyme transport and peristaltic activity was started after a resting period of at least 20 min following catheter placement. After identification of a phase 3 migrating motor complex, a standard test meal consisting of 500 g of commercially available yogurt with small pieces of fruit (450 kcal, 400 mL, 5.5 g fat, 12.5 g protein, 75 g carbohydrate) was administered, and data were collected for a further 2 h.

Statistical analysis

Impedance and manometry tracings were reconstructed on screen and the patterns were consecutively analyzed. Since we studied transduodenal bolus transport, only impedance signals related to a complete chyme transport over at least 6 cm beginning at the first impedance channel were included and analyzed as previously described as a bolus transport event (BTE)^[5,6]. This definition was used to exclude transpyloric movement of gastric contents into the duodenum bulb without initiation of duodenal peristalsis. BTE were classified according to (a) site of onset (proximal *vs* distal), (b) propulsion direction (propulsive *vs* retropropulsive), (c) propagation distance (short-spanned ≤ 8 channels or ≤ 16 cm *vs* long-spanned > 8 channels or > 16 cm), (d) number of components (simple = one

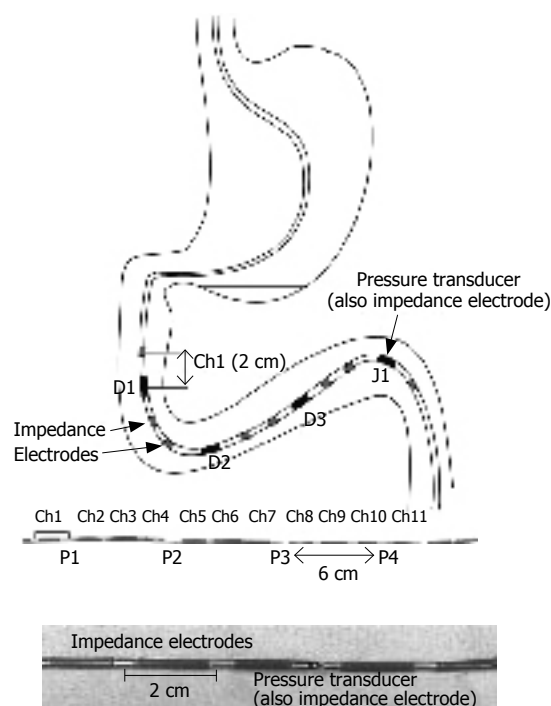


Figure 1 Modified manometry catheter for concurrent impedance-manometry procedure. The 4 semiconductor solid-state pressure transducers (P1-P4) serve also as impedance electrodes and are placed at 6 cm distance each. The 8 impedance electrodes (4 mm length) are arranged between the pressure transducers at a distance of 16 mm. Together with the pressure transducers, they form 11 impedance segments, each 2 cm long (Ch1-Ch11). The solid-state pressure transducers are located exactly between the impedance channels 1-2, 4-5, 7-8, and 10-11, respectively. The first channels were located at the first part of the duodenum.

component *vs* complex = multiple components). Thus the transport patterns were: (a) short-spanned propulsive, (b) retrograde, (c) long-spanned propulsive, and (d) complex propulsive^[5,6,8]. Of note, our previous validation study^[6] demonstrated that long-spanned BTE are associated with a significant drop of intraluminal pH and change of electrical conductivity, thus indicating real chyme movement originating from the stomach.

After identification and classification of the chyme transport patterns the corresponding peristaltic sequences were analyzed. Firstly, the peristaltic nature of the associated contractions was characterized as (a) stationary (isolated contraction observed in only one channel) or (b) propagated (contraction detected over 2, 3 or 4 pressure channels = 6, 12 and 18 cm). Secondly, propagated contractions were classified according to Summers *et al.*^[16] to be: (a) propagated contraction with single wave contraction (1 contraction), (b) propagated contraction with a double wave contraction (2 contractions) or propagated contraction with a clustered contraction (> 2 contractions occurring at a rate of 5 s) (c) propagated cluster of contractions (clustered contractions occurring at more than one pressure channel) as shown in Figure 2. A double spike wave was considered to be single wave. Contractions that were observed between the BTEs were not included for analysis. Data are expressed as total number of events counted.

RESULTS

Combined impedance-manometry during the interdigestive state

During the interdigestive phase 2 there were irregular motility activities as recorded by manometry and irregular chyme transport events as recorded by impedance-manometry (Figure 3, upper panel). Both impedance and manometry recorded the same features of the phase 3 complex (Figure 3, lower panel).

Combined impedance-manometry during the postprandial state

The postprandial manometry tracings showed irregular peristaltic activity, which is difficult to analyse (Figure 4, upper panel). However, with combined impedance and manometry the relationship between transduodenal bolus transport event and associated peristaltic activity could be investigated in more detail (Figure 4, middle panel). Plotting the tracings at high resolution, the patterns of chyme transport as recorded by impedance and of peristalsis as recorded by manometry were analysed systematically (Figure 4, lower panel).

Impedance bolus transport patterns

A total number of 564 BTEs were counted. Sixty seven BTEs (12%) were excluded, because they could not be clearly classified according to the impedance criteria. Of the remaining 497 BTEs the distribution of the impedance patterns was: (a) short-spanned propulsive transport, 110 events (22%), long-spanned propulsive transport, 307 events (62%), and (d) complex transport, 70 events (14%) and retrograde transport events, 10 (2%).

Relationships between impedance transport patterns and manometry peristaltic patterns

The relationship between impedance transport patterns and manometry peristaltic patterns could be classified as followed (Figure 5): (a) of the short-spanned propulsive chyme transports (110 events) the majority of them was associated either with a stationary contraction (43 events or 39%) or a single contraction propagated over only 2 pressure channels (64 events or 58%); (b) all long-spanned propulsive chyme transports (307 events) were associated with a propagated contractions; (c) all complex chyme transport patterns (70 events) were associated with propagated contractions, particularly over 4 pressure channels; (d) retrograde chyme transport was rare (10 events) and are associated either with a stationary contraction (7 events) or retrograde propagated single wave contraction (3 events).

Of the long-spanned bolus transport events (307 events), 92 BTEs (30% of them) were associated with a propagated contraction over 2 or 3 pressure channels (6-12 cm), and 215 BTEs (70% of them) were associated with a propagated contraction over 4 pressure channels (18 cm).

Of the long-spanned propulsive chyme transports over 4 channels (215 events), 61 events (28% of them) were associated with a propagated single wave contraction, and 154 events (72% of them) were associated with a propagated double wave contraction or a propagated

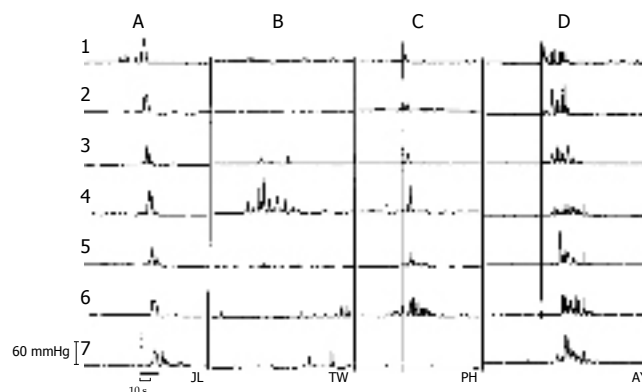


Figure 2 Classification of the peristaltic patterns according to Summers *et al*^[16]. The peristaltic pattern can be classified to be (A) propagated single (double spike) wave contraction, (B) isolated (stationary) cluster of contractions, (C) propagated contraction with a clustered contraction, and (D) propagated cluster of contractions.

contraction with a clustered contraction. None of these bolus transport was associated with a propagated cluster of contractions.

All complex propulsive chyme transports (70 events) were associated with propagated contractions over 4 channels (18 cm): 48 events (68% of them) were associated with a propagated double wave contraction or a propagated contraction with a clustered contraction, and 22 events (32% of them) were associated with a propagated cluster of contractions. None of these bolus transport was associated with a propagated single wave contraction.

Examples of the bolus transport events and their associated peristaltic patterns are shown in Figures 6-8.

DISCUSSION

Only few studies in man have directly analysed the spatial and temporal relationship between the patterns of chyme flow and patterns of peristaltic contraction waves in the duodenum, particularly during the postprandial state and in details. This study was performed to directly address this issue using the newly developed and validated technology of combined impedance and manometry for motility testing^[12-15]. The daisy-chained configuration of the impedance electrodes in the present system differ significantly from other systems^[17-19], where the impedance electrode pairs are located far from each other. As shown in previous impedance studies^[5,6] this catheter configuration offers a high spatial resolution for detailed monitoring of bolus transport patterns. The incorporated solid state pressure transducers allow the concurrent analysis of the corresponding contractile events.

As shown in Figure 3, the phase 3 complex is recorded identically by both techniques, showing the well-known characteristics of the migrating motor complex, similar to recent findings by Imam *et al*^[17]. During the postprandial phase, chyme transport and associated peristaltic activity can be accurately monitored, and thus a large number of bolus transport event (BTE) was obtained for detailed analysis.

Considering the manometry tracings alone, it has been

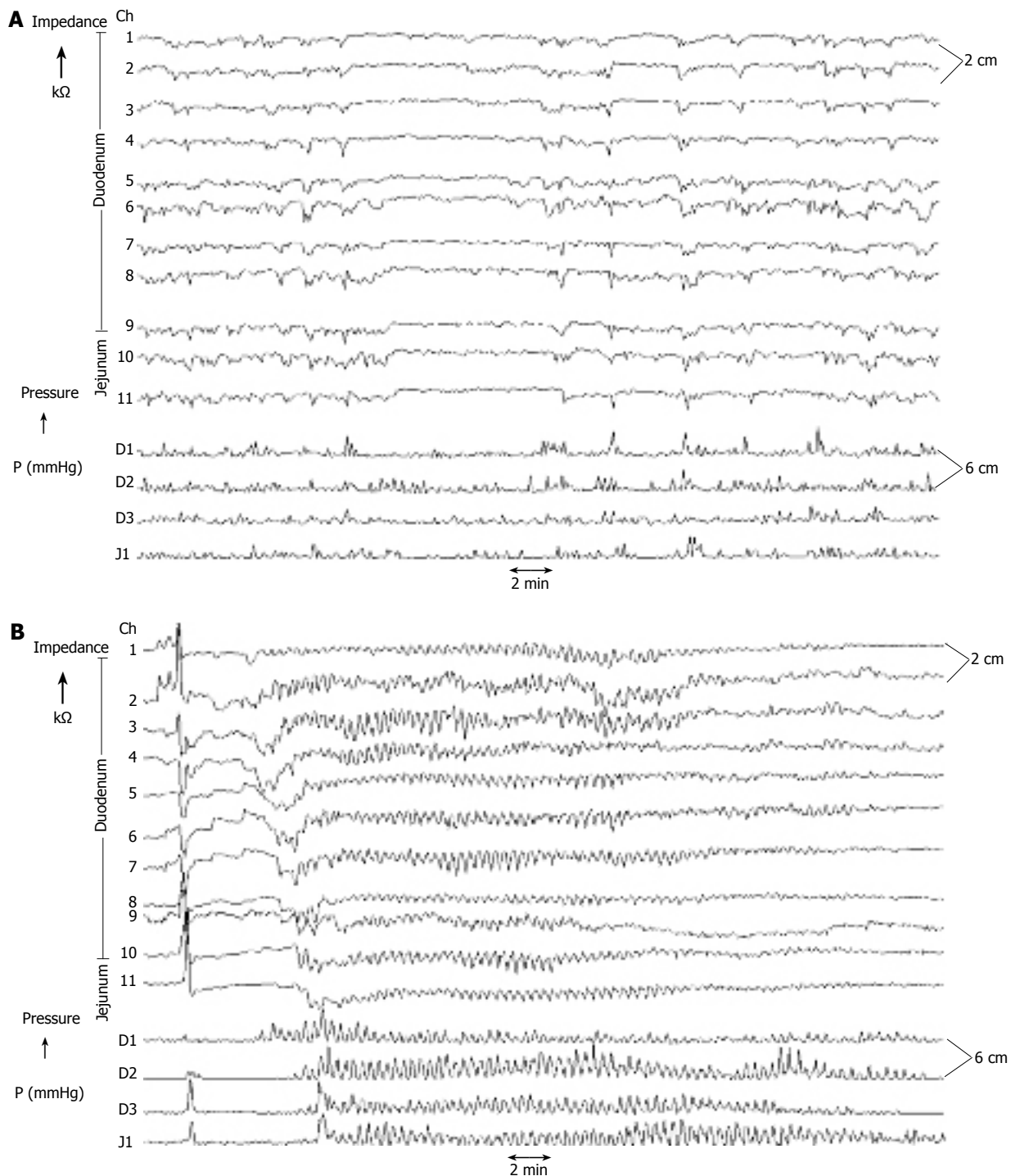


Figure 3 Concurrent Impedance Manometry (CIM) tracings. Upper panel: During the interdigestive phase irregular chyme transport at the impedance channels and irregular motor activities at the pressure channels are observed. Lower panel: A phase 3 complex displays nearly identical features with frequent changes of both pressure and impedance.

shown to be difficult to characterize contraction waves to be stationary or propagated^[20]. As shown in Figure 4 this problem can be overcome by the combined technique as shown in the recent study. After a bolus transport event (BTE) had been identified with impedance in the present study, the organization of the associated contraction waves could be analyzed. We did not include contraction waves that occurred between the bolus transport events, as compared to previous studies^[1,3,20], which analyzed the overall motility activity. Therefore, the results are not comparable. As only impedance events representing

a bolus transport over at least 6 cm were analysed, the present results provide data about the postprandial organization of peristaltic activity in association with a transduodenal bolus transport. Transpyloric chyme movement was not included in the present studies.

A major finding of the present study is the close relationship between transduodenal bolus transport patterns and peristaltic contraction patterns. The results showed that long-spanned chyme transport patterns are predominantly associated with propagated peristaltic patterns, whereas short-spanned chyme transport patterns

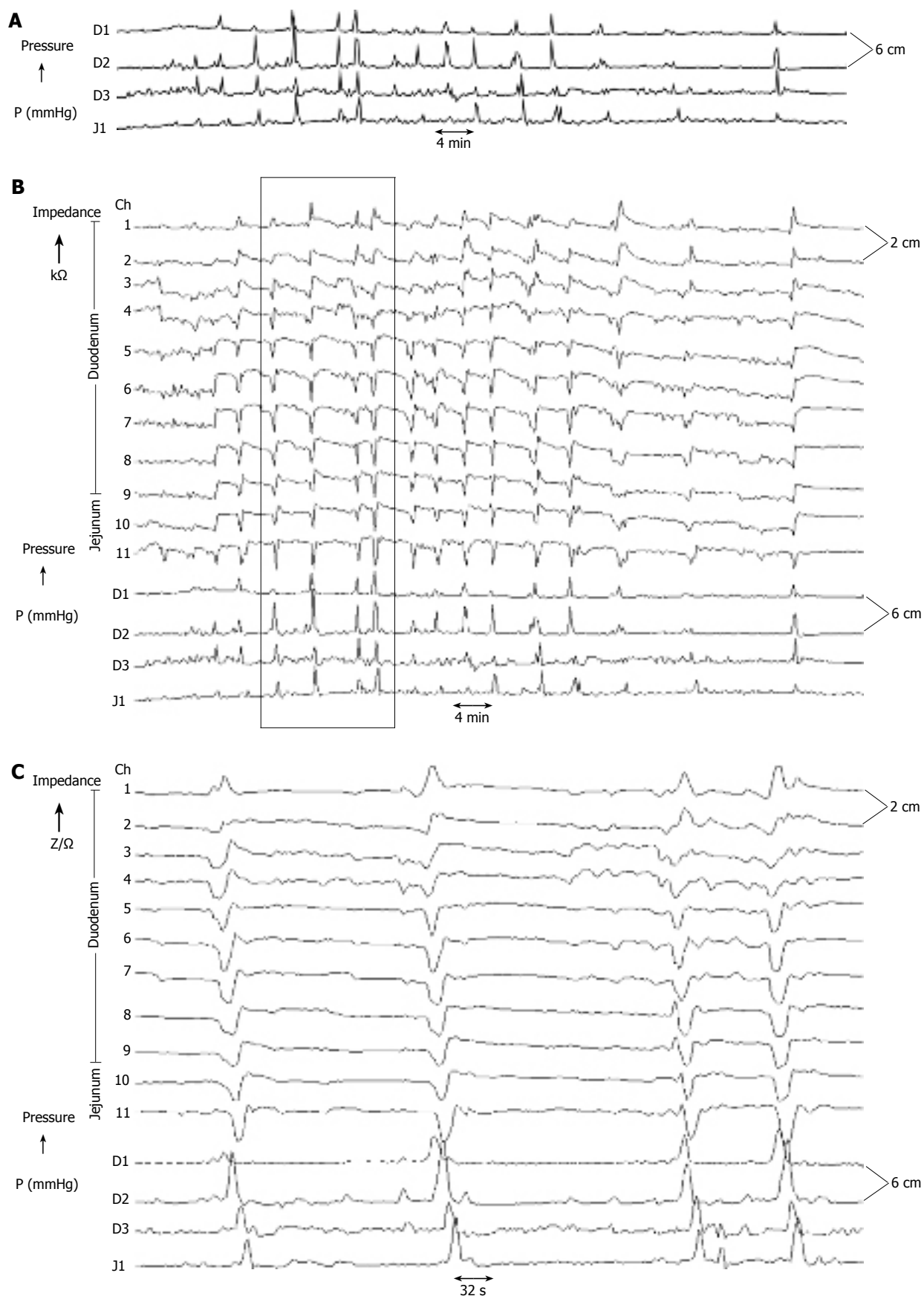


Figure 4 Concurrent Impedance Manometry (CIM) tracings after a test meal. Upper panel: Low time scaled manometry tracings of the postprandial state. Middle panel: Low time scaled impedance manometry tracings of the same period as above showing several bolus transport events with associated peristaltic activities. Lower panel: High time scaled impedance manometry tracings of the box allowing identification and classification of bolus transport patterns as well as analysis of associated peristaltic patterns.

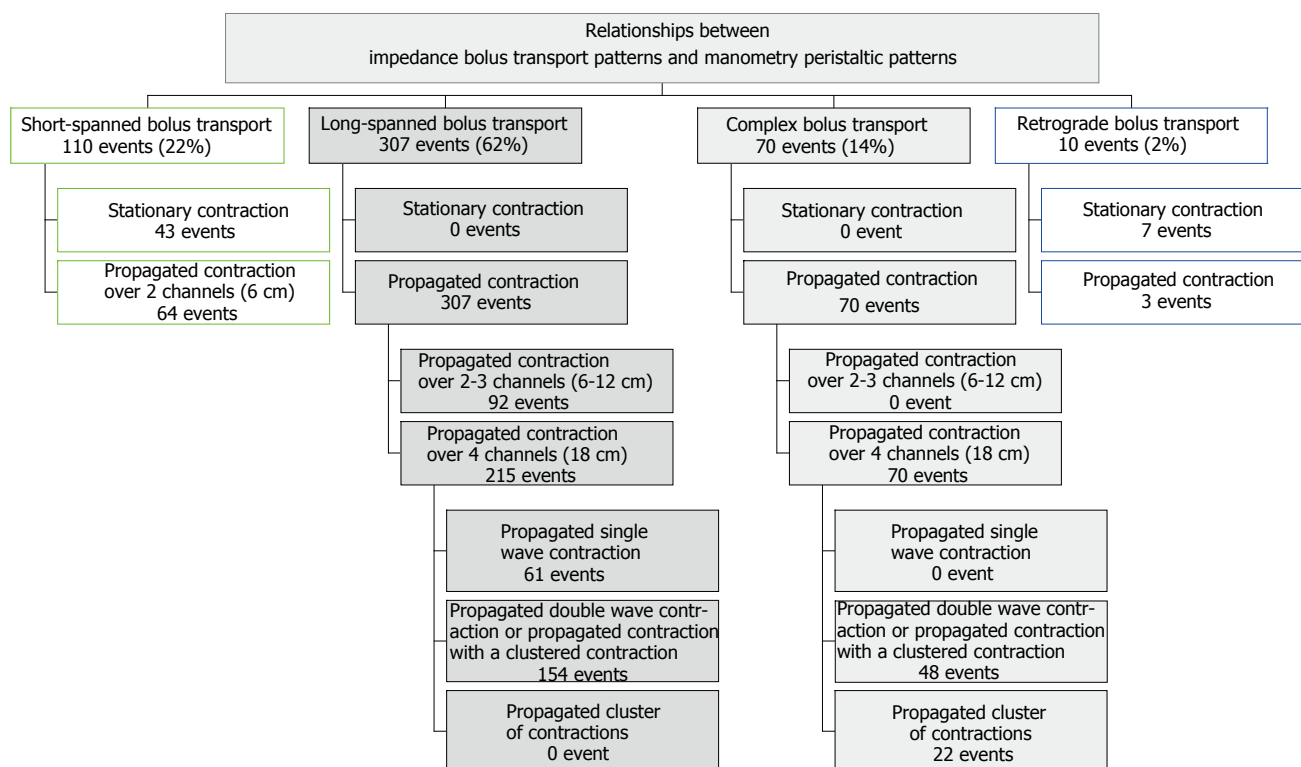


Figure 5 Distribution of bolus transport patterns and associated peristaltic patterns (see text for details).

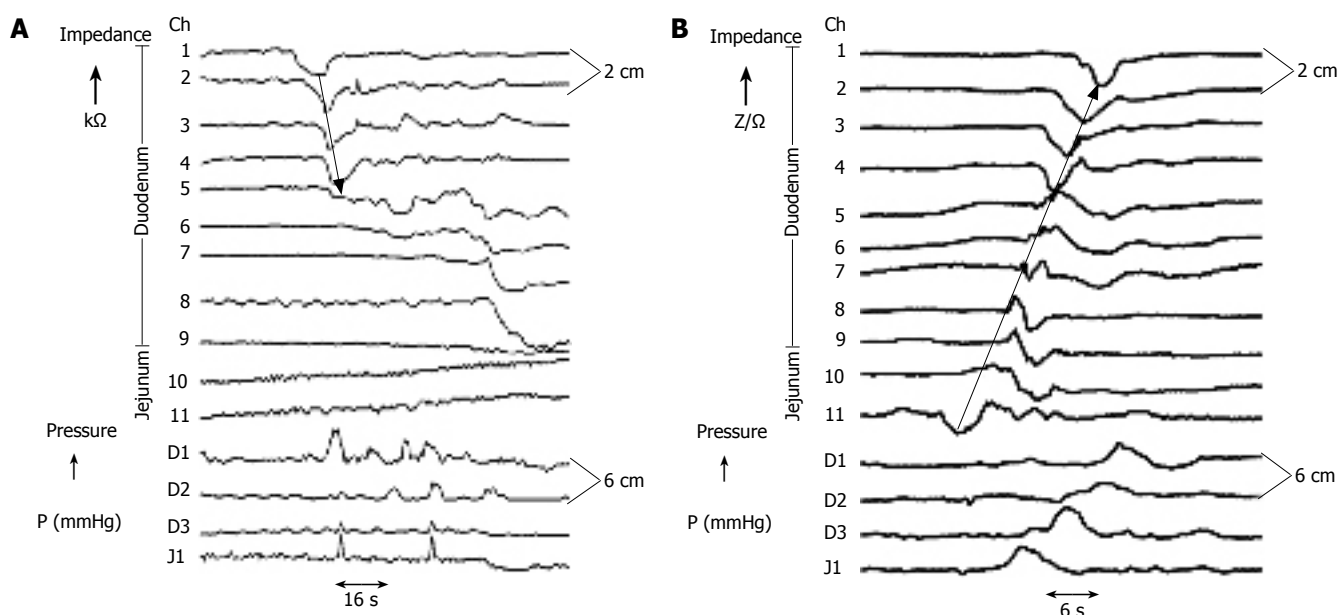


Figure 6 Examples of simple bolus transports and associated peristalsis. Upper panel: Short-spanned bolus transport with associated stationary contraction. Lower panel: Retrograde bolus transport with associated retrograde peristalsis.

are frequently related to stationary contractions or propagated contractions over short distance (6 cm). The data are consistent with results of a recent study^[17] showing that impedance corresponds better with fluoroscopic flows than manometry and that recording of pressure events can underestimate even flow events of substantial length. However, the quantitative data of this study are not comparable to ours because there are important differences with respect to study design and analysis algorithm: (a) in this study the impedance segments were spaced 5 cm apart

from each other as compared to the cascade configuration in our study, thus, the recording of impedance patterns differs substantially; (b) all impedance events with a drop of 12% or more below baseline, even if detected in only one impedance segment (spread distance < 5 cm), were included as compared to impedance signals of a complete bolus passage over 6 cm in our study. Since an impedance drop only indicates arrival of a bolus front but not always a complete bolus passage, it might represent transpyloric chyme movement into the proximal duodenum, which is

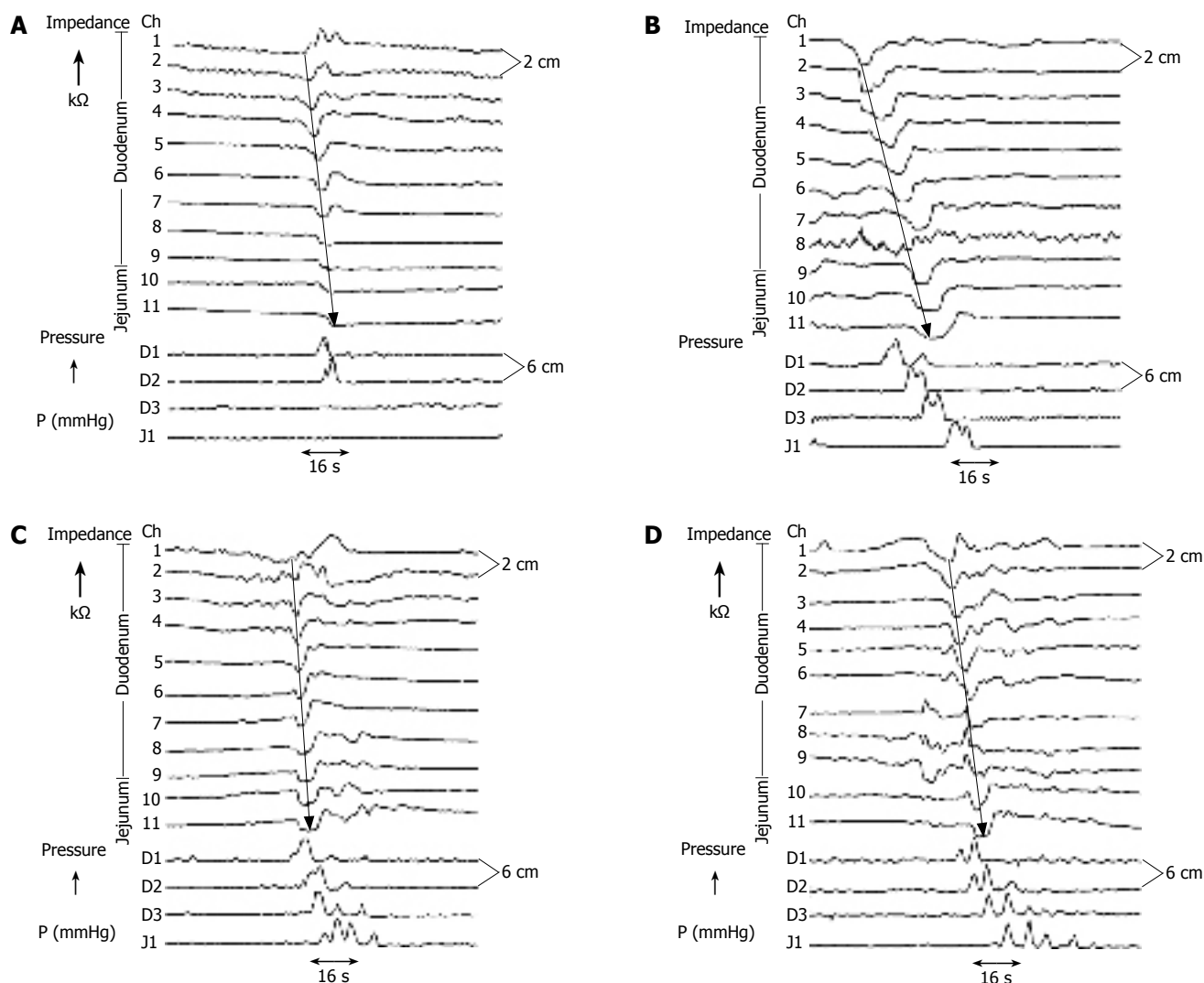


Figure 7 Examples of long-spanned bolus transports and associated peristalsis. (A) A long-spanned bolus transport with the bolus front traversing the whole duodenum. The associated peristaltic sequence displayed a propagated single wave contraction from D1 to D2. This peristaltic pattern was classified to be simple (propagated single wave contraction). (B) A long-spanned bolus transport traversing the whole duodenum. The associated peristaltic sequence displayed a propagated contraction with a double wave contraction at channel D1 and double spike contractions at D2, D3 and J1. This peristaltic pattern was classified to be complex (propagated contraction with a double wave contraction). (C) A long-spanned bolus transport with the bolus front traversing the whole duodenum. The associated peristaltic sequence displayed single wave contractions at channel D1-D3 and a clustered contractions at J1. This peristaltic pattern was classified to be complex (propagated contraction with a clustered contraction). (D) A long-spanned bolus transport with the bolus front traversing the whole duodenum. The associated peristaltic sequence displayed double wave contractions at channel D1 and a clustered contractions at D2, D3 and J1. This peristaltic pattern was classified to be complex (propagated cluster of contractions).

detected at the first duodenal channel as compared to a transduodenal bolus transport over at least 6 cm.

Another major finding is related to the motor mechanisms regulating transduodenal bolus transport. Distinct peristaltic patterns have been identified. Our data showed, that if a bolus is propelled over a long distance (> 4 pressure channels or 18 cm), it is frequently associated with a complex peristaltic sequence, which can be either a propagated contraction with double wave contraction, a propagated contraction with a clustered contraction, or rarely, a propagated cluster of contractions. The finding that cluster of contractions (double wave contraction, clustered contraction) are an integral part of a peristaltic sequence associated with a transduodenal bolus transport is new. The retrospective analysis of these contraction patterns without the associated impedance tracings frequently fails to clearly identify the clustered contractions to be a part of bolus-associated peristaltic sequence. These results may

explain the facts that the analysis and classification of all contraction waves in human small intestine is difficult, and most of them have been classified to be stationary^[20].

The dominant patterns of propulsive bolus transports with associated propulsive peristaltic patterns support the existence of a precise spatial and temporal organization of the contraction waves in human duodenum during the postprandial state^[21-24]. Two recent studies investigating the relationships between antral contraction, transpyloric fluid flow and duodenal motility observed that transpyloric fluid transport is associated with duodenal propagation^[18,21]. The results of the present study strongly support previous reports showing that coordinated duodenal contraction waves are an important determinant regulating antroduodenal chyme flow^[22,24,25], as well as gastric emptying^[26].

The recent finding of different propulsive bolus transport patterns associated with different propulsive

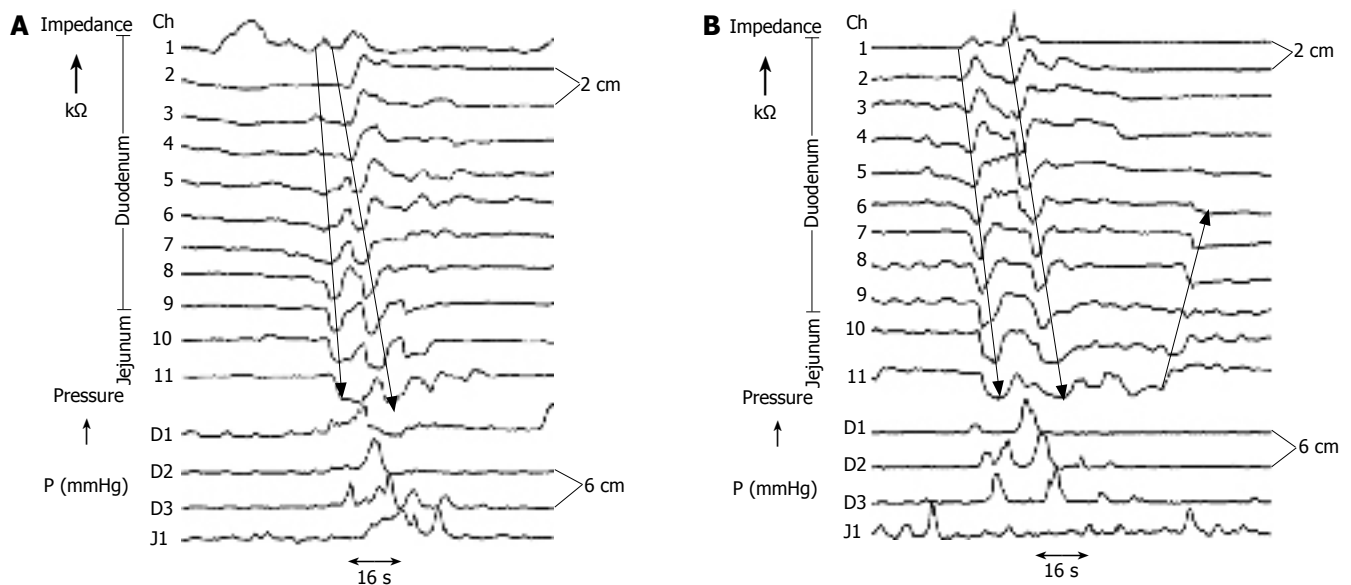


Figure 8 Examples of complex bolus transports and associated complex peristalsis. (A) A complex bolus transport with two components propelling with different propulsion velocity as illustrating by the arrows. The associated peristaltic sequence is complex showing a long lasting double spike contraction at D1, a single wave contraction at D2, a clustered contraction at D3 and a multispike double wave contraction at J1. (B) A complex bolus transport comprising two boluses following each other very rapidly as illustrating by the anterograde arrows. The associated peristalsis showed two separate peristaltic sequences which seemed to be connected together by an interpolate contraction wave at D2. A retrograde bolus movement (retrograde arrow) seemed to be derive from a retrograde peristaltic sequence with a single contraction wave seen at J1.

peristaltic patterns underlines the physiological difference between the duodenum and the esophagus, where the bolus transport patterns are highly uniform and the peristaltic sequences are predominantly simple^[7,13]. This finding support the view that the duodenum is not only a conduit but also an active segment, which is able to generate contractions transporting and mixing gastric contents together with duodenal juices into the jejunum^[27-31]. Since previous studies^[32,33] indicated that contraction patterns in the duodenum are quite different from those in the jejunum, further studies should examine, if similar bolus transport patterns and peristaltic patterns will be found in other segments of the gut.

The answers about the question regarding how complex peristaltic sequences can be regenerated during a bolus transport can be sought in results of electrophysiological studies. Intestinal motility is considered to be controlled by interaction between myogenic, neural and humoral factors^[34-35]. In recent studies several motility patterns have been characterized as peristaltic or pendular, stationary or propagating, or twitch or segmental^[36-37]. Furthermore, several electrical signals have been shown to be associated with different types of contractions including slow waves, spikes, or bursts^[36-38]. Therefore, peristalsis with associated propagated peristaltic waves should not be regarded as a simplex reflex, but rather as a co-ordinated locomotor pattern, which can be induced either by fluid distension, local stretch, or mucosal stroking^[36]. Careful examination of the spatial and temporal relationship between spontaneous slow waves and peristaltic waves showed that they seem to constitute two separate electrical events that may drive two different mechanisms of contractions^[39]. Slow waves are not in rhythm with peristaltic waves and they may occur in different groupings and patterns. These waves may travel in the same or in opposite directions from each other and may

propagate in the oral or caudal direction, and therefore, may modulate each other. Similarly, slow waves and spikes seem to be propagated by different mechanisms through different cell networks^[32]. Thus, the spatial and temporal characteristics of contraction in the small intestine seem to be determined not only by the direction of the slow wave but also whether or not spikes are generated after these slow waves^[32,40].

There are 2 limitations of the recent studies, which should be evaluated in further studies: (a) since the composition of the test meals significantly affect small intestine motility, it remains to be determined, if the recent bolus transport and peristaltic patterns will be the same by using different test meals; (b) since the intertransducer distance significantly affect the recognition of propagated pressure waves, it remains to be determined, if more closely spaced recording points with 1-2 cm apart may provide more accurate data.

In summary, combined impedancemetry and manometry in human duodenum provides detailed data about the relationship between the organization of contraction waves and the patterns of chyme flow during the postprandial state. This technique improves the analysis of intestinal motility. Several postprandial peristaltic patterns associated with transduodenal bolus transport have been identified showing that cluster of contractions constitutes an integral part of the peristaltic sequence associated with transduodenal bolus transport. The results provide new insights into the peristaltic mechanisms that are associated with transduodenal chyme transport and maintain the physiological function of the duodenum. The present results clearly indicate that comprehensive motility testing in the small intestine, particularly during the postprandial state, should be performed using the combined technique. The present data will serve as basis

findings forwarding clarifying small intestinal motor dysfunction.

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Comparative clinical trial of S-pantoprazole *versus* racemic pantoprazole in the treatment of gastro-esophageal reflux disease

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Abstract

AIM: To compare the efficacy and tolerability of S-pantoprazole (20 mg once a day) versus racemic Pantoprazole (40 mg once a day) in the treatment of gastro-esophageal reflux disease (GERD).

METHODS: This multi-centre, randomized, double-blind clinical trial consisted of 369 patients of either sex suffering from GERD. Patients were randomly assigned to receive either one tablet (20 mg) of S-pantoprazole once a day (test group) or 40 mg racemic pantoprazole once a day (reference group) for 28 d. Patients were evaluated for reduction in baseline on d 0, GERD symptom score on d 14 and 28, occurrence of any adverse effect during the course of therapy. Gastrointestinal (GI) endoscopy was performed in 54 patients enrolled at one of the study centers at baseline and on d 28.

RESULTS: Significant reduction in the scores (mean and median) for heart burn ($P < 0.0001$), acid regurgitation ($P < 0.0001$), bloating ($P < 0.0001$), nausea ($P < 0.0001$) and dysphagia ($P < 0.001$) was achieved in both groups on d 14 with further reduction on continuing the therapy till 28 d. There was a statistically significant difference in the proportion of patients showing improvement in acid regurgitation and bloating on d 14 and 28 ($P = 0.004$ for acid regurgitation; $P = 0.03$ for bloating) and heart burn on d 28 ($P = 0.01$) between the two groups, with a higher proportion in the test group than in the reference group. Absolute risk reductions for heartburn/acid regurgitation/bloating were approximately 15% on d 14 and 10% on d 28. The relative risk reductions were 26%-33% on d 14 and 15% on d 28. GI endoscopy showed no significant difference in healing of esophagitis ($P = 1$) and gastric erosions ($P = 0.27$) between the two groups. None of the patients in either group reported any adverse effect during the course of therapy.

CONCLUSION: In GERD, S-pantoprazole (20 mg) is more effective than racemic pantoprazole (40 mg) in improving symptoms of heartburn, acid regurgitation, bloating and equally effective in healing esophagitis and gastric erosions. The relative risk reduction is 15%-33%. Both drugs are safe and well tolerated.

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Key words: Gastro-esophageal reflux disease; Pantoprazole; Efficacy; Tolerability

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INTRODUCTION

The primary treatment goals in patients with gastroesophageal reflux disease (GERD) are relief of symptoms, prevention of symptom relapse, healing of erosive esophagitis, and prevention of complications of esophagitis^[1]. Proton pump inhibitors (PPIs) block the final step in acid production and hence are the most effective inhibitors of acid secretion^[2]. Recent advances in analytical methods for the separation of enantiomers of PPIs have led to a considerable interest in the stereoselective pharmacokinetics of PPI enantiomers^[3].

Pantoprazole, a selective and long acting PPI, is a chiral sulfoxide that is used clinically as a racemic mixture of S-pantoprazole and R (+) pantoprazole. The pharmacokinetics of R and S isomers of pantoprazole vary widely in extensive and poor metabolizers^[4]. The use of a single isomer avoids this variation and offers predictable pharmacokinetics. Animal studies have shown that S-pantoprazole is more potent (1.5 to 1.9 times) and effective (3 to 4 times) than racemate in inhibiting gastric lesions in different pre-clinical models^[3,5], suggesting that in human patients 20 mg of S-pantoprazole would be at least equivalent in efficacy to 40 mg of racemic pantoprazole. The present study was to compare the efficacy and tolerability of S-pantoprazole (20 mg) *versus* racemic pantoprazole (40 mg) in the treatment of gastro-oesophageal reflux disease (GERD) in adult patients.

MATERIALS AND METHODS

Materials

S-pantoprazole was synthesized by Emcure Pharmaceuticals Ltd. Tablets of S-pantoprazole (20 mg) and racemic pantoprazole (40 mg) were provided by the same source in coated opaque packets containing 28 tablets each without labeling the identity of the contents.

Methods

This multi-centre, randomized, double blind comparative study permitted by Drugs Controller General of India (DCGI) was conducted in compliance with the 'Guidelines for Clinical Trials on Pharmaceutical Products in India-GCP Guidelines' issued by the Central Drugs Standard Control Organization, Ministry of Health, Government of India (<http://www.cdscn.in/html/GCP1.html>). The Ethical Committee approval was taken from Sharada Clinic Ethical Committee, Karad for Dr. S Erram, Independent Ethical Committee for Dr. H Thacker, Independent Ethical Committee of Surya Hospital, Pune for Dr. V Pai, Dr. V Mandora and Dr. J Shinde. Written informed consent was obtained from the subjects. The study was initiated on November 7, 2004 and completed on February 25, 2005.

Subjects

Patients of either sex, aged 18-65 years, with clinically confirmed GERD, were enrolled in the study after providing written informed consent. Patients with known hypersensitivity to pantoprazole and any major hematologic, hepatic, metabolic, gastrointestinal or endocrine disorder requiring any other anti-GERD medication and women who were pregnant or lactating or child bearing potential and not practicing effective method of contraception were excluded from the study.

Patients were randomized in blocks of ten, as per the computer generated randomization chart (www.randomization.com) into two treatment groups: one group receiving 20 mg S-pantoprazole once daily for 28 d (test group) while the other group receiving 40 mg racemic pantoprazole once daily (reference group) for the same period. The identity of the treatment allocated was unknown to either investigators or patients. The reference and test medications had a similar appearance, shape and size and could not be distinguished from each other based on their external appearance. All the patients completed the 28 d therapy during which they were followed up twice on d 14 and 28. Medication compliance was checked by counting the number of tablets left in the packet, if any.

Scoring of symptoms

Heartburn, regurgitation and dysphagia are symptoms of GERD. Although nausea and bloating are symptoms of dyspepsia which can be an overlapping condition in patients with GERD, this study included patients with symptoms of nausea and bloating also in order to capture the overall clinical benefit. The severity of heartburn, acid regurgitation, bloating and nausea was scored as follows: 0 = none, no symptom; 1 = mild, occasional symptoms that did not affect normal activities; 2 = moderate, frequent symptoms or symptoms that affected normal activities; 3

Table 1 Baseline demographic variables

Variable	Reference group	Test group
<i>n</i>	182	187
Male: Female	115:67	114:73
Mean age \pm SD (yr)	42.3 \pm 11.7	42 \pm 12.3

= severe, constant symptoms. The severity of dysphagia was scored as follows: 0 = normal; 1 = occasional sticking of solids; 2 = swallowing semisolids and Pureed food; 3 = swallowing liquids only. Scores were recorded for all the patients at the initiation of the study and then on d 14 and 28. Improvement in symptoms was defined as reduction in baseline symptom score. Assessment of within-group efficacy was done by comparing symptom scores on d 14 and 28 with baseline value on d 0 of the study. Assessment of between-group efficacy was done by comparing the proportions of patients showing improvement in symptoms in each group. GI endoscopy was done in 54 patients enrolled at one of the study centers on d 0 and 28. Tolerability profile was assessed by comparing the incidence of possible drug-related adverse effects in each group on d 14 and 28 of the study.

Statistical analysis

The results were analyzed using Fisher's exact test (two sided) for difference in proportions, Friedman's test (non-parametric repeated measures ANOVA) for comparison of between-days score (variation amongst column medians). For this, $P < 0.05$ was considered statistically significant. Individual differences between columns (d 0 vs d 14 and d 0 vs d 28) were assessed by Dunn's multiple comparisons test. The GraphPad InStat (version 3.06, 32 bit for Windows, September 11, 2003, GraphPad Software, San Diego California USA, www.graphpad.com) and WINPEPI suite (Abramson JH (2004) WINPEPI (PEPI-for-Windows) computer programs for epidemiologists as well as Epidemiologic Perspectives & Innovations (2004, 1:6) softwares were used for statistical analysis. UBC clinical significance calculator (from <http://www.healthcare.ubc.ca/calc/clinsig.html>) was used to calculate the absolute risk reduction (ARR), relative risk reduction (RRR) and number needed to treat (NNT). ARR is the absolute arithmetic difference in rates of bad outcomes between experimental and control participants in a trial, calculated as experimental event rate minus control event rate. RRR is the proportional reduction in rates of bad outcomes between experimental and control participants in a trial, calculated as experimental event rate minus control event rate divided by control event rate. NNT is the number of patients who need to be treated to achieve one additional favorable outcome, calculated as $1/ARR$.

RESULTS

Three hundred and sixty-nine patients (229 males and 140 females) with symptoms of GERD were enrolled in the study after providing written informed consent. Baseline demographic variables of the two groups are shown in Table 1. There was no statistically significant difference between the two groups at baseline.

Table 2 Efficacy in the reference (racemic pantoprazole 40 mg, $n = 182$) and test (S-pantoprazole 20 mg, $n = 187$) groups

Symptoms		d 0		d 14		d 28		P^3
		Ref	Test	Ref	Test	Ref	Test	
Heart burn	Mean \pm SD	1.8 \pm 0.8	1.9 \pm 0.8	1.2 \pm 0.8	1.2 \pm 0.9	0.6 \pm 0.9	0.6 \pm 0.8	< 0.0001
	Median (Percentile 25 th , 75 th)	2 (1.2)	2 (1.3)	1 (1.2) ²	1 (0.5.2) ¹	0 (0.1) ²	0 (0.1) ¹	
Regurgitation	Mean \pm SD	1.6 \pm 0.9	1.7 \pm 0.9	1.1 \pm 0.8	1 \pm 0.8	0.5 \pm 0.8	0.4 \pm 0.6	< 0.0001
	Median (Percentile 25 th , 75 th)	2 (1.2)	2 (1.2)	1 (0.2) ²	1 (0.2) ¹	0 (0.1) ²	0 (0.1) ¹	
Bloating	Mean \pm SD	1.4 \pm 0.9	1.4 \pm 1	0.9 \pm 0.9	0.8 \pm 0.8	0.5 \pm 0.8	0.3 \pm 0.6	< 0.0001
	Median (Percentile 25 th , 75 th)	1 (1.2)	2 (1.2)	1 (0.2) ²	1 (1.2) ¹	0 (0.1) ²	1 (1.1) ¹	
Nausea	Mean \pm SD	1.4 \pm 1	1.3 \pm 1	0.8 \pm 0.9	0.7 \pm 0.8	0.4 \pm 0.8	0.4 \pm 0.7	< 0.0001
	Median (Percentile 25 th , 75 th)	2 (0.2)	1 (0.2)	1 (0.2) ²	0 (0.1) ¹	0 (0.0.25) ²	0 (0.1) ¹	
Dysphagia	Mean \pm SD	0.3 \pm 0.6	0.5 \pm 0.8	0.2 \pm 0.6	0.2 \pm 0.5	0.1 \pm 0.4	0.1 \pm 0.4	< 0.001
	Median (Percentile 25 th , 75 th)	0 (0.0)	0 (0.1)	0 (0.0)	0 (0.0) ¹	0 (0.0)	0 (0.0) ¹	

^{1,2} Dunn's multiple comparisons test; ³P value for ANOVA (Friedman's test).

Table 3 Between-group efficacy in improvement of symptoms

Decrease compared to baseline (d 0) in score	Patients showing improvement in symptoms (n)											
	Heart burn				Regurgitation				Bloating			
	On d 14		On d 28		On d 14		On d 28		On d 14		On d 28	
	Ref	Test	Ref	Test	Ref	Test	Ref	Test	Ref	Test	Ref	Test
Decrease by 1	89	93	56	71	90	111	76	86	62	81	69	65
Decrease by 2	9	21	61	67	6	12	52	52	11	14	36	38
Decrease by 3	0	1	11	15	0	2	8	20	3	2	9	21
Patients who improved (n)	98	115	128	153	96	125	136	158	76	97	114	124
Patients with symptoms (n)	172	179	172	179	165	170	165	170	149	143	149	143
Fisher's test, P	0.19		0.01		0.004		0.004		0.004		0.03	

No significant differences were present in the baseline symptom scores in both groups. In both treatment groups, significant reductions in the mean and median scores for heart burn ($P < 0.0001$), acid regurgitation ($P < 0.0001$), bloating ($P < 0.0001$), nausea ($P < 0.0001$) and dysphagia ($P < 0.001$) were achieved on d 14 with further reduction on continuing the therapy till d 28 (Table 2). The percentage of patients in the reference group, achieving improvement in heart burn, acid regurgitation, bloating, nausea and dysphagia was 57.0%, 58.2%, 51.0%, 66.4% and 58.6% respectively at the end of 14 d therapy, and 74.4%, 82.4%, 76.5%, 81.3% and 79.3% respectively at the end of 28 d therapy. The percentage of patients in the test group, achieving improvement in heart burn, acid regurgitation, bloating, nausea and dysphagia was 64.3%, 73.5%, 67.8%, 72.7% and 67.8% respectively at the end of 14 d of therapy, and 85.5%, 92.9%, 86.7%, 81.3% and 84.7% respectively at the end of 28 d of therapy. There was a statistically significant difference in the proportion of patients showing improvement in acid regurgitation and bloating on d 14 and 28 ($P = 0.004$ for acid regurgitation; $P = 0.03$ for bloating), and heart burn on d 28 ($P = 0.01$) between the two groups, with a higher proportion in the test group than in the reference group (Table 3, Figure 1).

Of the 54 patients who underwent GI endoscopy, baseline findings of esophagitis and erosions were present in 17 (68%) and 6 (2.4%) patients out of 25 patients in the reference group and 21 (72.4%) and 2 (0.7%) patients out of 29 patients in the test group respectively. Twenty-eight days after therapy, esophagitis and erosions were present in 7 (28%) and 6 (2.4%) patients out of 25 patients in the

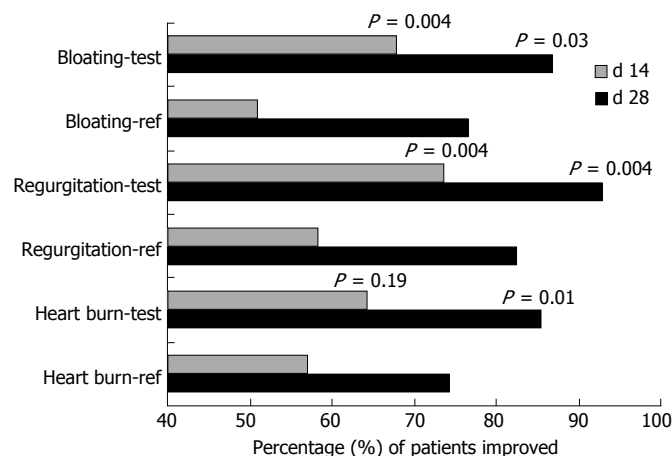


Figure 1 Percentage of patients showing improvement in heartburn, acid regurgitation and bloating in two treatment groups on d 14 and 28.

reference group and 9 (31%) and 3 (1.03%) patients out of 29 patients in the test group respectively. There was no significant difference in both treatment groups in healing of esophagitis ($P = 1$) and erosions ($P = 0.27$) (Table 5).

None of the patients in either group reported any adverse effect during the course of the study. Both drugs were well tolerated.

DISCUSSION

All PPIs are chiral compounds. Chirality can introduce marked selectivity and specificity into the way the drug is handled by the body and how the compound interacts

Table 4 Absolute risk reduction (95% CI for differences between proportions), relative risk reduction (RRR) and number needed to treat (NNT)

Symptoms, d	Absolute risk reduction ¹ (95% CI for difference in improvement) (%)	RRR (%)	NNT
Heartburn, d 28	+ 11.1 (2.7-19.4)	15	9
Acid regurgitation, d 14	+ 15.3 (5.3-25.0)	26	7
Acid regurgitation, d 28	+ 10.5 (3.6-17.5)	13	10
Bloating, d 14	+ 16.8 (5.7-27.9)	33	6
Bloating, d 28	+ 10.2 (1.4-19.0)	13	10

¹Significant differences in favor of test group were analyzed.

with the receptor or enzyme binding sites in some cases. Overall, this may lead to variations in pharmacokinetic and pharmacodynamic properties and differences in safety and toxicity profiles^[6].

The present study showed that symptoms of GERD *viz* heart burn, acid regurgitation, bloating, nausea and dysphagia improved significantly in both treatment groups. The results also showed that a significantly higher proportion of patients treated with 20 mg S-pantoprazole achieved improvement in heart burn, acid regurgitation and bloating as compared to patients treated with 40 mg racemic pantoprazole (Table 3, Figure 1). The absolute risk reduction was approximately 15% on d 14 and 10% on d 28. The relative risk reduction was approximately 15% on d 28 and at least 26% on d 14. This translates to a NNT of approximately 6-7 patients on d 14 and 9-10 patients on d 28 (Table 4). The findings of GI endoscopy showed that 20 mg S-pantoprazole was equally effective compared to 40 mg racemic pantoprazole in healing esophagitis ($P = 1$) and gastric erosions ($P = 0.27$). Both drugs were well tolerated with no adverse effects. These findings confirm that S-pantoprazole is the more active component of racemic pantoprazole that can be used even at half the dose of racemate to achieve a better efficacy in the treatment of GERD.

Table 5 Findings of GI endoscopy

	Number of patients with findings (n)			
	d 0		d 28	
	Esophagitis	Erosions	Esophagitis	Erosions
Ref	17	6	7	6
Test	21	2	7	3
Fisher's test, P	0.77	0.12	1	0.27

In summary, 20 mg S-pantoprazole is more effective than 40 mg racemic pantoprazole in improving symptoms of heart burn, acid regurgitation, bloating of GERD. Both drugs are safe and well tolerated.

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Short mucin 6 alleles are associated with *H pylori* infection

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Abstract

AIM: To investigate the relationship between mucin 6 (MUC6) VNTR length and *H pylori* infection.

METHODS: Blood samples were collected from patients visiting the Can Tho General Hospital for upper gastrointestinal endoscopy. DNA was isolated from whole blood, the repeated section was cut out using a restriction enzyme (*Pvu* II) and the length of the allele fragments was determined by Southern blotting. *H pylori* infection was diagnosed by ¹⁴C urea breath test. For analysis, MUC6 allele fragment length was dichotomized as being either long (> 13.5 kbp) or short (≤ 13.5 kbp) and patients were classified according to genotype [long-long (LL), long-short (LS), short-short (SS)].

RESULTS: 160 patients were studied (mean age 43 years, 36% were males, 58% *H pylori* positive). MUC6 *Pvu* II-restricted allele fragment lengths ranged from 7 to 19 kbp. Of the patients with the LL, LS, SS MUC6 genotype, 43% (24/56), 57% (25/58) and 76% (11/46) were infected with *H pylori*, respectively (*P* = 0.003).

CONCLUSION: Short MUC6 alleles are associated with *H pylori* infection.

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Key words: *H pylori*; Mucin 6; Polymorphism; Variable number of tandem repeats

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INTRODUCTION

H pylori has the unique ability to colonize the human stomach. Infection with *H pylori* invariably leads to gastritis and in many instances to peptic ulcer disease^[1]. Additionally, *H pylori* infection has been associated with gastric cancer^[2]. It is a common infection throughout the world, with prevalence ranging from below 20% in developed countries to over 80% in developing countries. Some risk factors for *H pylori* infection have been identified, such as low socio-economic status or poor hygiene^[3]. However, there is remarkable inter-individual variability in susceptibility to the infection that cannot be explained by differences in environmental factors.

Another factor that may be related to *H pylori* infection susceptibility is the composition of the mucus gel layer in the stomach, in which *H pylori* resides. This layer protects the underlying epithelium from acid, proteases, mechanical trauma, and pathogenic micro-organisms and its main constituents are high molecular weight glycoproteins named mucins. These mucins consist of a polypeptide backbone with O-linked oligosaccharide side chains, which largely determine the properties of the mucins^[4]. Interestingly, there is substantial inter-individual variation in the number of these side chains. This is caused by a variable number tandem repeat (VNTR) polymorphism in the genes encoding for the mucins. VNTRs consist of repeated DNA sequences and the number of repeats is highly variable. The resulting repeated amino acid sequences are located in the central part of the mucin polypeptide backbone, to which the oligosaccharide side chains are attached^[5]. Therefore, this polymorphism leads to the production of mucin polypeptides that substantially differ in both length and glycosylation^[6,7]. Thus, this VNTR polymorphism may affect the protective properties of the mucins and consequently the susceptibility to *H pylori* infection.

Normal gastric mucosa is characterized by the expression of mucins MUC1, MUC5AC and MUC6. MUC1 is of the membrane-bound type, whereas MUC5AC and MUC6 are of the secreted, gel-forming type^[8]. MUC6 and MUC1 show extensive VNTR variation,

MUC5AC only moderate^[6,9]. Furthermore, the length of the repeated sequence differs: a single MUC6 tandem repeat sequence consists of 507 base pairs whereas single MUC1 and MUC5AC tandem repeat sequences consist of only 60 and 24 base pairs, respectively^[6]. Therefore, the VNTR polymorphism has the most profound impact on allele length and protein structure of MUC6.

Despite the significant structural consequences of these VNTR polymorphisms, few studies investigated their pathophysiological consequences. In a study comparing gastric cancer patients with healthy blood donors, shorter VNTR sections were associated with gastric cancer for MUC6^[10] and MUC1^[11]. This effect may be mediated by an altered susceptibility to *H. pylori* infection which is an important factor in gastric carcinogenesis, since Vinall *et al*^[9] showed that short VNTR sections were associated with *H. pylori* infection for MUC1. However, there are no data available regarding the relationship between *H. pylori* infection and VNTR polymorphism in MUC6, which is abundant in the stomach and has the most extensive VNTR variation^[12].

Therefore, the aim of this study was to investigate the hypothesis that susceptibility to *H. pylori* infection is related to MUC6 VNTR length. We studied a sample of 160 patients referred for upper gastrointestinal endoscopy and found that patients with short MUC6 allele fragments have a significantly higher risk of being infected with *H. pylori*, which suggests that mucin 6 protein length modifies susceptibility to *H. pylori*.

MATERIALS AND METHODS

Subjects

From September to December 2003, all patients with upper gastrointestinal symptoms visiting the Gastroenterology Outpatient Clinic of the Can Tho General Hospital for upper gastrointestinal endoscopy were asked to participate in this study. Patients who had not been treated for *H. pylori* infection in the past and who gave written informed consent were included in the study. At baseline, data regarding age, gender, smoking habits and alcohol consumption were registered.

Diagnosis of *H. pylori* infection

All patients had a ¹⁴C urea breath test (Heliprobe™, Noster system AB, Stockholm, Sweden). Patients were not allowed to use proton pump inhibitors/H₂-receptor antagonists or antibiotics during the two weeks preceding the breath testing. After an overnight fast patients took a HeliCap™ capsule (containing 1 μCi of ¹⁴C urea) with 50 mL of water. Ten minutes later, a breath sample was collected (BreathCard™) and analyzed during 4 min. Measuring more than 50 counts was regarded as proof of *H. pylori* infection, measuring fewer than 25 counts was regarded as proof of absence of *H. pylori* infection^[13].

Determination of MUC6 allele length

Blood samples were collected for DNA isolation (Puregene™ kit, Gentra systems, Minneapolis, USA). MUC6 allele fragment length was measured using Southern blot

analysis. The DNA samples were digested with the *Pvu*II restriction enzyme as described previously by Vinall *et al*^[6]. This enzyme cuts just outside the tandem repeat domain and clearly reveals the MUC6 VNTR polymorphism. The resulting DNA fragments were separated by agarose gel electrophoresis (0.7% agarose in 0.04 mol/L Tris, 0.001 mol/L EDTA, pH 8.0) at 35 V for 18 h. We used λ -Hind III digest as a marker of DNA fragment length. DNA fragments were then transferred onto nylon membranes (Gene Screen Plus™ hybridization transfer membrane, Boston, USA). Afterwards, the nylon membranes were treated with ultraviolet radiation and prepared for hybridization by Church buffer. The probe, which consisted of two MUC6 tandem repeats, was produced by polymerase chain reaction with forward primer 5'-ACCTCTTTGGT-GACTCCAATTA-3' and reverse primer 5'-AACGT-GAGTGGGAAGTGTGGT-3' and randomly labelled with α -³²PdCTP. The resulting PCR product was verified by sequencing. After 18 h of hybridization of the probe in 0.5 mol/L phosphate buffer containing 7% SDS and 0.001 mol/L EDTA, SSPE/SDS-solutions were used to remove non-specifically bound probe. Using the λ -Hind III digest as a reference, the individual MUC6 allele fragment lengths were calculated.

Statistical analysis

The primary outcome of this study was the presence or absence of *H. pylori* infection. For analysis, MUC6 allele fragment length was dichotomized as being either short (≤ 13.5 kb) or long (> 13.5 kb) and patients were classified according to genotype [long-long (LL), long-short (LS), short-short (SS)]. Baseline characteristics for *H. pylori* positive and negative patients were compared. MUC6 genotype and baseline characteristics were related to *H. pylori* infection by means of unadjusted and adjusted logistic regression analyses, using the SAS® statistical software package (SAS Institute Inc., USA). Statistical significance was defined as a $P < 0.05$. Missing values were excluded from analysis.

RESULTS

Population

During the study period, 160 patients [mean age 43 years, 58 (36%) males, 92 (58%) *H. pylori* infected] were included. Table 1 shows that *H. pylori* positive and negative patients were comparable for all baseline characteristics except age.

MUC6 allele fragment length

Using the restriction enzyme *Pvu*II, a clear length polymorphism was detected. Minimum fragment length difference was 0.5 kb, reflecting the length of a single MUC6 tandem repeat that consists of 507 base pairs. MUC6 was found to be highly polymorphic with *Pvu*II-restricted fragment lengths ranging from 7 to 19 kb [mean 13.8 kb (SD: 2)] (Figure 1).

Factors associated with *H. pylori* infection (Table 2)

Mean MUC6 allele fragment length was shorter for *H. pylori* positive patients than for *H. pylori* negative patients (13.4

Table 1 Baseline characteristics of *H pylori* positive and negative patients

Characteristic	<i>H pylori</i> positive <i>n</i> (%) (<i>n</i> = 92)	<i>H pylori</i> negative <i>n</i> (%) (<i>n</i> = 68)
Mean age (SD) (yr)	46 (12) ^a	39 (13)
Gender		
Male	29 (50)	29 (50)
Female	63 (62)	39 (38)
Currently smoking		
Yes	22 (56)	17 (44)
No	70 (58)	51 (42)
Current alcohol consumption		
Yes	16 (55)	13 (45)
No	76 (58)	55 (42)

^a*P* < 0.05 vs *H pylori* negative.

Table 2 Factors associated with *H pylori* infection

Factor	Unadjusted analysis		Adjusted analysis ¹	
	Odds ratio	95% CI	Odds ratio	95% CI
MUC6 genotype (SS vs LS/LL) ^a	3.18	1.5-6.9	2.93	1.3-6.5
Age group (> 45 vs ≤ 45 yr) ^a	2.11	1.1-4.1	2.11	1.0-4.3
Gender (male vs female)	0.62	0.3-1.2	0.47	0.2-1.9
Currently smoking	0.94	0.5-2.0	1.46	0.5-4.3
Current alcohol consumption	0.89	0.4-2.0	1.39	0.5-3.9

^a*P* < 0.05. ¹Adjusted for MUC6 genotype, age group, gender, current smoking and current alcohol consumption.

vs 14.2, *P* = 0.001). Since there were too many different allele fragment lengths to analyse separately, MUC6 allele fragment length was dichotomized as being either short (≤ 13.5 kb) or long (> 13.5 kb). Furthermore, patients were grouped according to genotype [long-long (LL), long-short (LS), short-short (SS)].

Patients with two short allele fragments were more often infected with *H pylori* than patients with one long and one short allele fragment [Odds ratio (95% CI): 2.41 (1.0-5.7)] or patients with two long allele fragments [4.24 (1.8-10.0)]. Figure 2 shows that there seems to be a gradual increase in prevalence of *H pylori* infection from 43% (24/56) for patients with two long allele fragments, through 57% (25/58) for patients with one long and one short allele fragments, to 76% (11/46) for patients with two short allele fragments.

Additionally, Table 2 shows that of the other patient characteristics measured, only age was associated with *H pylori* infection, and that the influence of MUC6 genotype remained virtually unchanged after adjusting for age group, gender, smoking and alcohol consumption.

DISCUSSION

The aim of this study was to investigate the relationship between MUC6 VNTR polymorphisms and *H pylori* infection. We were able to confirm that MUC6 VNTR length is

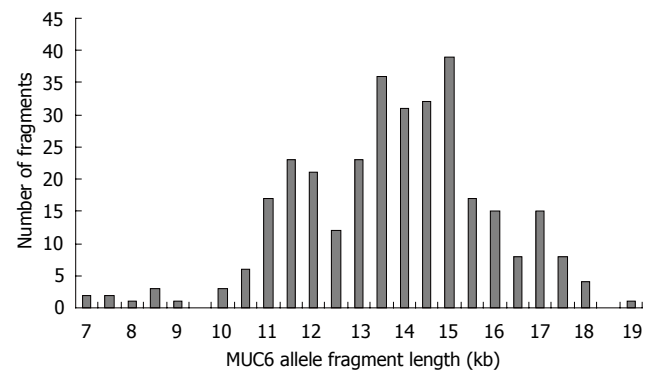


Figure 1 Distribution of MUC6 allele fragment lengths after *Pvu* II restriction enzyme digestion.

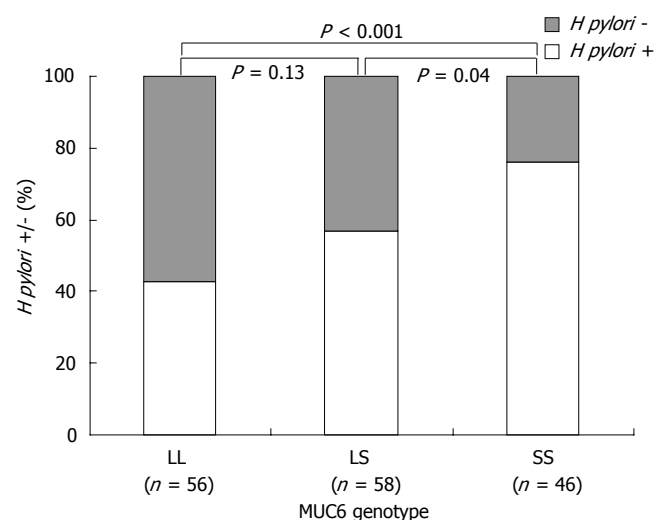


Figure 2 Percentage of *H pylori* infected patients according to MUC6 genotype.

highly polymorphic and our data suggest that *H pylori* infection is more frequent in patients with short MUC6 alleles.

Few other studies investigated the MUC6 VNTR polymorphism. Vinall *et al*^[6] found 11 different allele fragment lengths for MUC6, ranging from 8 to 13.5 kb. This degree of variation is considerably lower than in our study. It is unlikely that this difference in VNTR length variation is inherent to the Southern blotting technique, as we used the same restriction enzyme. However, the aforementioned study used the Centre d'Étude du Polymorphisme Humain (CEPH) series of families in France and these may be much more homogeneous than our study population.

The relationship between *H pylori* infection and mucin VNTR length has been investigated for another mucin gene, MUC1, and the results were similar to ours. Like other mucins, MUC1 lubricates epithelial structures and constitutes a barrier against acid, proteases and pathogenic organisms. Vinall *et al*^[9] showed that short MUC1 alleles were associated with *H pylori*-induced gastritis. Therefore, MUC1 and MUC6, although arising from different families of mucins, may be involved in the same mechanism regarding *H pylori* infection^[14].

Our results are also compatible with research focusing

on the relationship between mucin allele length and gastric cancer. In a study investigating 157 gastric cancer patients, it was found that short MUC6 alleles were more frequent in patients with gastric cancer than in healthy blood donors^[10]. This seems to be in line with our results that short MUC6 alleles are associated with *H pylori* infection. In fact, because *H pylori* has been classified as a class I carcinogen, the higher prevalence of *H pylori* infection among patients with short MUC6 alleles may (partly) explain the higher prevalence of gastric cancer in these patients. Again, the same goes for MUC1 since Carvalho *et al*^[11] stated that short MUC1 alleles were associated with gastric cancer. More research is necessary to determine whether these relationships are independent.

However, other researchers claimed that MUC5AC, and not MUC6, is important in *H pylori* infection: Van den Brink *et al*^[13] stated that *H pylori* co-localised with MUC5AC but not with MUC6, and Van de Bovenkamp *et al*^[6] stated that MUC5AC, and not MUC6, was the most important receptor for *H pylori*. However, in the study by Van den Brink *et al*, antibodies recognizing the MUC6 precursor rather than mature MUC6 were used. Therefore, it seems plausible that the precursor MUC6 is only found in neck and gland cells, where MUC6 is synthesized. However, the mature MUC6, which is secreted, may be found throughout a much larger area. In fact, Ho *et al*^[17] recently confirmed that the mucin within the glands consisted entirely of MUC6, but they also showed that, although the mucus layer on the gastric surface consisted primarily of MUC5AC, layers of MUC6 were interspersed between the layers of MUC5AC.

Regarding the receptor function of the mucins, MUC5AC is the primary source of Lewis B (Le^b), a terminal carbohydrate chain that acts as a ligand for the bacterial adhesion molecule BabA^[16,18]. However, although other receptor sites may be present on MUC6^[19], mucins may be involved in many other processes besides bacterial binding.

In fact, recently, Kawakubo *et al*^[20] showed that secretions from the glands (consisting of MUC6) may have an antibiotic effect on *H pylori*, while secretions from the superficial epithelium (primarily consisting of MUC5AC) may have a pro-biotic effect, thereby limiting *H pylori* infection to the superficial epithelium and protecting the deeper layers of the gastric mucosa. This is consistent with the geographical distribution of *H pylori* described by Van den Brink *et al*^[15]. The antibiotic effect was mediated by terminal α -1, 4-linked *N*-acetylglucosamine (α -1,4-GlcNAc) residues, which are present on the variable region of MUC6. Furthermore, the presentation of multiple terminal α -1,4-GlcNAc residues as a cluster may be important for achieving optimal activity. This may explain our finding that shorter MUC6 molecules, which have fewer α -1,4-GlcNAc residues and therefore lower antimicrobial activity, are associated with *H pylori* infection. More research is necessary to further elucidate the functions of the mucins.

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RAPID COMMUNICATION

Resistance to activated protein C is a risk factor for fibrostenosis in Crohn's disease

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<http://www.wjgnet.com/1007-9327/12/6026.asp>

Abstract

AIM: To evaluate the effect of resistance to activated protein C (aPCR), the most common known inherited thrombophilic disorder, on the risk of intestinal operation of fibrostenosis in patients with Crohn's disease (CD).

METHODS: In a previous study, we assessed the prevalence of aPCR in CD. In a retrospective case-controlled study, 8 of these CD patients with aPCR were now compared with 24 CD patients without aPCR, matched by gender, age at diagnosis and duration of disease in a 1:3 fashion. The primary end point was the occurrence of an intestinal CD-related operation with evidence of fibrostenosis in the bowel resection specimen.

RESULTS: The Kaplan-Meier analysis revealed that patients with aPCR had a lower probability of remaining free of operation with fibrostenosis than patients without aPCR ($P = 0.0372$; exact log-rank test) resulting in a significantly shorter median time interval from diagnosis of CD to the first operation with fibrostenosis (32 vs 160 mo). At 10 years, the likelihood of remaining free of operation with fibrostenosis was 25% for patients with aPCR and 57.8% for patients without aPCR.

CONCLUSION: CD patients with aPCR are at higher risk to undergo intestinal operation of fibrostenosis than those without aPCR. This supports our hypothesis of aPCR being a possible risk factor for fibrostenosis in CD.

INTRODUCTION

Fibrostenotic lesions of the bowel are frequent and serious complications in Crohn's disease (CD). They often require surgery, which may lead to short bowel syndrome in case of repeated bowel resections^[1-4]. Intestinal fibrosis in CD as a consequence of chronic transmural inflammation is characterized by increased production of extracellular matrix, including fibrillar collagen, by activated mesenchymal cells, such as fibroblasts and smooth muscle cells^[5,6]. However, the exact pathophysiological mechanism explaining variable disposition of CD patients to develop fibrostenotic lesions is unknown. But some risk factors have been discovered. For example, NOD2/CARD15 variants^[7-9] and smoking have been associated with fibrostenosis^[10,11]. Moreover, CD patients with antibody responses against *Saccharomyces cerevisiae* (ASCA), CD-related bacterial sequence (I2), *E. coli* outer membrane porin C (anti-OmpC), and neutrophil nuclear antigen (pANCA) have also been reported as being more likely to develop fibrostenotic lesions^[12-15].

Resistance to activated protein C (aPCR)^[16], representing the most common known inherited thrombophilic disorder^[17], has been shown to increase the rate of fibrosis in other inflammatory diseases, such as chronic viral hepatitis^[18]. Activated protein C serves as a natural anticoagulant by cleavage and inactivation of factor V a. Factor V plays a central regulatory role in hemostasis, since it contributes to the conversion of prothrombin to active thrombin, which, on the other hand, transforms fibrinogen into fibrin. A single-point mutation in the gene encoding factor V, also known as factor V Leiden, results in a form of factor Va that is resistant to degradation by activated protein C, leading to a relative hypercoagulation state^[19].

The increase of the rate of fibrosis in chronic viral hepatitis caused by aPCR was explained by thrombotic obliterations of small portal and hepatic veins resulting in local hepatocyte death and increased development of fibrosis and by the effect of thrombin through activation of stellate cells, which are the mediators of fibrosis in the liver^[20]. However, the role of aPCR in the development of fibrostenotic lesions in CD has not been elucidated till now. The potential role of the clotting system in CD has already been shown. Granulomatous vasculitis, intravascular fibrin deposition and capillary microthrombi have been reported in CD lesions^[21,22]. Additionally, CD has been observed less frequently in patients with inherited haemophilic disorders^[23]. On the other hand, aPCR as a thrombophilic disorder may favor intravascular fibrin deposition and thrombotic obliteration of the small vessels of intestinal lesions in CD, resulting in increased local cell death and fibrosis.

We, therefore, hypothesized that aPCR increases the rate of fibrosis in CD patients. We expected that CD patients with aPCR are more likely to have fibrostenotic lesions of the bowel than patients without aPCR, resulting in a lower probability of remaining free of operation with fibrostenosis.

MATERIALS AND METHODS

Patients and study design

This is a retrospective case-control study on the influence of aPCR on the presence of fibrostenotic lesions found at intestinal CD-related operations in CD patients. In a previous study on risk factors for thromboembolism in inflammatory bowel disease (IBD), we assessed the prevalence of aPCR in patients with IBD, including 77 patients with CD^[24]. We had detected 8 CD patients with aPCR who were now compared with CD patients without aPCR selected from the same database. CD patients with aPCR ($n = 8$) and without aPCR ($n = 24$) were matched by gender, age at diagnosis (± 10 years) and duration of disease (± 3 years) in a 1:3 fashion. A colleague blinded for the clinical course of the patients performed the matching. All patients included in the study were Caucasians and had been in routine care at our institution (Medical University of Vienna, Department of Internal Medicine IV, Division of Gastroenterology and Hepatology, Vienna). The diagnosis of CD was based on established criteria of clinical, radiological, endoscopic, or histological findings^[25]. The location of CD was determined according to the Vienna classification^[26]. The information on the clinical course, smoking habits, and the medical and surgical management was reviewed from the charts of the patients. A "smoker" was defined as a patient who had smoked at least seven cigarettes weekly for at least one year^[11]. Immunosuppressants, such as azathioprine, were considered for analysis if the duration of treatment had been at least 3 mo.

The primary end point of the study was the presence of fibrostenosis found at intestinal CD-related operations documented by the pathological and/or surgical report, respectively. The secondary end point was the occurrence of intestinal CD-related operations regardless of fibroste-

nosis. Only intestinal operations, such as bowel resections, stricturoplasties and gastrointestinal bypass-surgery, were included in the analysis. Creations of a stoma, intestinal reconstructions, exploratory laparotomies, and surgery of complicated perianal CD were excluded from the analysis. Two observers blinded for the aPCR status of the patients (W.R. and J.P.) reviewed the pathological and surgical reports of the patients for the presence of fibrostenosis. The Ethics Committee of the Medical University of Vienna approved this study.

Definition of fibrostenotic lesions

Strictureing disease is defined by the Vienna classification as the occurrence of constant luminal narrowing demonstrated by radiologic, endoscopic, or surgical examination combined with prestenotic dilatation and/or obstructive signs or symptoms but without evidence of penetrating disease^[26]. In our retrospective analysis, for the purpose of defining fibrostenotic lesions at the time of surgery, we modified this definition for four reasons. First, strictures of the bowel in CD may result not only from fibrostenotic lesions but also from inflammatory obstruction without fibrosis. Second, internal fistulas are often associated with strictures and such mechanical factors may even favor the development of fistula, which might explain the coexistence of fibrostenotic lesions and perforation^[27]. Third, radiologic, endoscopic, and surgical examination may report contradictory results. The most reliable data on complicated disease are available after resection from surgical and pathological reports. In these reports, fibrostenotic lesions were defined as luminal narrowing and bowel wall thickening on naked-eye examination of the surgical resection specimen. Histologically, strictures were recognized by thickening of the muscularis mucosa and by fibrosis of the submucosa^[27]. And fourth, we excluded "obstructive symptoms" from the definition, since it may be difficult to differentiate clinically from other disease-related complications (e.g. inflammation, fistula and abscess).

We, therefore, defined fibrostenotic lesions as the occurrence of bowel wall thickening and luminal narrowing on naked eye examination in the pathological and surgical report found at intestinal CD-related surgery as well as the occurrence of thickening of the muscularis mucosa and of fibrosis of the submucosa in the histological part of the pathological report. In case of discrepancies between pathological and surgical examination, the pathological report of the surgical resection specimen was rated with higher priority than the surgical report. In case of stricturoplasties and gastrointestinal bypass surgery, if a surgical resection specimen was not available, the assessment of fibrostenosis was based solely on the surgical report. The diagnosis of a fibrostenotic lesion was irrespective of evidence of penetration.

Assay system

aPCR was determined as described previously using the assay Coatest[®] aPCTM Resistance (Chromogenix, Mölndal, Sweden) according to the manufacturer's instructions^[24]. The normal range of the aPC ratio was > 1.9 and aPCR, therefore, was diagnosed if the aPC ratio was ≤ 1.9 . This

Table 1 Characteristics of 32 patients with Crohn's disease (frequencies, %, median values with range)

	With aPCR (n = 8)	Without aPCR (n = 24)
Sex ratio (M/F)	3/5	9/15
Age at diagnosis (yr)	30 (13-53)	31 (10-46)
Duration of disease (mo)	140 (86-257)	145 (65-272)
aPC ratio ¹	1.52 (1.19-1.64)	2.31 (2.08-2.66)
Patients with VTE in the history	5 (63%) ^b	2 (8%)
Smokers	4 (50%)	19 (79%)
Patients under azathioprine	4 (50%)	15 (63%)
Location of CD ²		
Terminal ileum	2 (25%)	4 (17%)
Colon	1 (12.5%)	5 (21%)
Ileocolon	4 (50%)	12 (50%)
Upper gastrointestinal tract	1 (12.5%)	3 (12%)

aPCR: resistance to activated protein C; CD: Crohn's disease; VTE: venous thromboembolism. ¹The patients were divided into the two groups according to the value of the aPC ratio: \leq (diagnosis of aPCR) and > 1.9 (normal range), respectively. ²The location of disease was classified according to the Vienna classification^[26]. ^b $P < 0.01$ vs patients without aPCR.

test with predilution of samples with factor V-deficient plasma has been shown to have a very high sensitivity and specificity of nearly 100% for the diagnosis of factor V Leiden, including a discrimination between heterozygous and homozygous subjects^[28-30].

Statistical analysis

All computations were performed with the use of SAS software, version 9 (SAS Institute Inc., Cary, NC, USA). Data were presented as frequencies, percentages and median values with range, respectively. Differences between groups were analyzed using the fisher's exact test for categorical data and the Mann-Whitney *U*-test for continuous data. Survival time methods were used to analyze the time from diagnosis of CD to the first intestinal CD-related operation with fibrostenosis and from diagnosis of CD to the first intestinal CD-related operation, respectively, (uncensored observations) or the duration of follow-up among patients without intestinal CD-related operation with fibrostenotic lesions and without intestinal CD-related operation, respectively (censored observations)^[31]. The probability of survival free of intestinal CD-related operation with fibrostenosis and survival free of intestinal CD-related operation regardless of the presence of fibrostenosis, respectively, was estimated according to the Kaplan-Meier method^[32]. Differences between Kaplan-Meier curves of both groups were analyzed using the exact log-rank test for unequal follow-up^[33]. The study design included 1:3 matching (CD with aPCR : CD without aPCR) with regard to gender, age at diagnosis and duration of CD. Differences were considered significant if P was < 0.05 .

RESULTS

Study population

The clinical data of both patient groups are summarized

Table 2 Intestinal CD-related surgery in 32 patients with Crohn's disease (frequencies, %, median values)

	With aPCR (n = 8)	Without aPCR (n = 24)
Patients having undergone an operation ¹	6 (75%)	16 (67%)
Patients having undergone an operation with fibrostenosis ²	6 (75%)	13 (54%)
Patients with fibrostenosis at 1 st operation ²	6/6 (100%)	10/16 (62.5%)
Location of fibrostenosis at 1 st operation ³		
Terminal ileum	4	8
Colon	2	0
Upper gastrointestinal tract	0	2
Median time from diagnosis of CD to 1 st operation ¹ (mo)	32	80.5
Median time from diagnosis of CD to 1 st operation with fibrostenosis ² (mo)	32 ^a	160

aPCR: resistance to activated protein C; CD: Crohn's disease. ¹Operations: intestinal CD-related operations; ²Operations with fibrostenosis: intestinal CD-related operations with fibrostenosis; ³The location of fibrostenosis was classified according to the Vienna classification for location of Crohn's disease^[26]. ^a $P < 0.05$ vs patients without aPCR.

in Table 1. Eight patients had an aPC ratio below 1.9 and were diagnosed as having aPCR: one patient had an aPC ratio of 1.19 consistent with homozygous for factor V Leiden and the other 7 patients had aPC ratios ranging from 1.46 to 1.64 consistent with heterozygous for factor V Leiden^[30]. Twenty-four patients had values of the aPC ratio within the normal range. Neither patient group differed in terms of location of disease, and percentage of smokers and patients who had been treated with azathioprine (Table 1). Furthermore, the number of cigarettes per day, the duration of smoking, and the dosage and duration of treatment with azathioprine did not differ between either patient group (data not shown). No immunosuppressants other than azathioprine had been administered. None of the patients had received infliximab or any other biological agent. Patients with aPCR had a history of venous thromboembolic complications diagnosed by imaging procedures significantly more often than the patients without aPCR ($P < 0.01$). Most of the thromboembolic events had been deep venous thromboses of the legs and pulmonary emboli.

Intestinal CD-related surgery

Six of 8 patients with aPCR and 16 of 24 patients without aPCR underwent intestinal surgery. Altogether, 44 intestinal CD-related operations (13 in patients with aPCR and 31 in patients without aPCR) had been performed. All operations had been intestinal resections, except for one case of gastroentero-anastomosis, which was necessary to circumvent the obstructed duodenum in a patient with CD of the upper gastrointestinal tract. A strictureplasty and a resection of another bowel segment performed at the same surgical procedure in a patient with aPCR were counted as one operation. The location of the bowel resections did not differ between both patient groups and was most likely of the terminal ileum (Table 2). In addition to bowel resections, three balloon dilations of fibrostenotic

lesions had been performed in two patients with aPCR but not in patients without aPCR. These interventions were not included in the analysis.

Operations with fibrostenosis: primary end point

There was a clear tendency for fibrostenosis to be found more often in the presence of aPCR. At the first intestinal CD-related operation, fibrostenotic lesions were found in all 6 (100%) patients with aPCR, but only in 10 out of 16 (62.5%) patients without aPCR ($P = 0.079$). The Kaplan-Meier analysis revealed that patients with aPCR had a significantly lower probability of remaining free of operation with fibrostenosis compared to patients without aPCR ($P = 0.0372$; exact log-rank test). This was also represented by the median time from diagnosis to the first operation with fibrostenosis being significantly shorter in patients with aPCR than in patients without aPCR (32 *vs* 160 mo; Table 2). In a further step, the calculation was limited to 120 mo, since this time interval was just below the median observation time of the patients (140 and 145 mo, respectively) and, therefore, more than half of the patients were under follow-up during the first 10 years after diagnosis of CD ($P = 0.0216$; exact log-rank test) (Figure 1). At 10 years, the likelihood of remaining free of operation with fibrostenosis was 25% for patients with aPCR and 57.8% for patients without aPCR.

Both observers who reviewed the pathological and surgical reports of the patients for fibrostenotic lesions agreed on the presence or absence of fibrostenosis in all operations. In 4 out of 44 operations, a fibrostenosis was described in the pathological report but was not mentioned in the surgical report. In all these four cases (1 patient with aPCR and 3 patients without aPCR), the surgeons described inflammatory masses that made it difficult to diagnose fibrostenosis in the surgical situation.

Overall operations: secondary end point

Concerning any intestinal CD-related operation without regard to whether it revealed fibrostenosis or not, patients with aPCR did not undergo operations more often than patients without aPCR. Six of 8 (75%) patients with aPCR and 16 of 24 (67%) patients without aPCR had undergone intestinal surgery ($P = 0.32$) (Table 2). Furthermore, the median time interval from diagnosis of CD to the first operation (Table 2), and the probability of remaining free of intestinal surgery (Kaplan-Meier survival curve not shown) did not significantly differ between the two patient groups ($P = 0.24$).

DISCUSSION

To our best of knowledge, this is the first report on an association between resistance to activated protein C and fibrostenosis in Crohn's disease. In a case-controlled study setting, aPCR increased the risk of fibrostenotic lesions found at intestinal surgery as described by surgical and/or pathological reports. CD patients with aPCR had a significantly lower probability of remaining free of intestinal operations with fibrostenosis. These data support our hypothesis that aPCR accelerates the development of

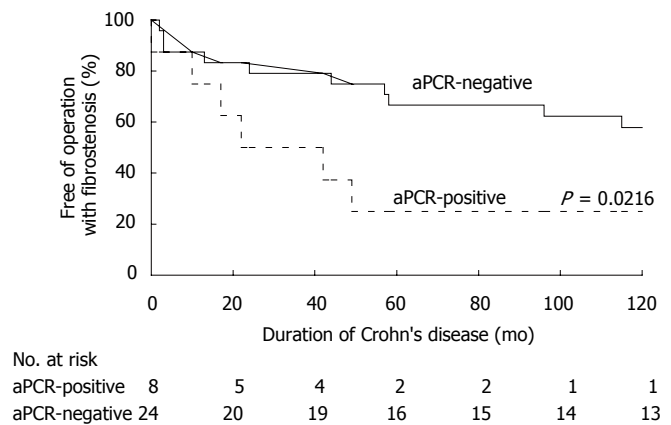


Figure 1 Kaplan-Meier curves for CD patients with and without aPCR, for remaining free of intestinal CD-related operation with fibrostenosis. A significantly lower proportion of patients with aPCR remained free of operation with fibrostenosis as compared to CD patients without aPCR ($P = 0.0216$; exact log-rank test). The calculation was limited to a period of 120 mo.

fibrosis in intestinal lesions in CD patients.

The primary end-point in the present study was the occurrence of an intestinal CD-related operation with fibrostenosis. Fibrostenotic lesions were referred to bowel wall thickening and luminal narrowing on naked-eye examination and to patho-histological features. This definition of fibrostenosis was modified from the definition of disease behavior used in the Vienna classification^[26] and was irrespective of evidence of penetration. The most important reasons for modification were the association of internal fistula and fibrostenosis in multiple bowel resection specimens^[27] as well as the possible occurrence of inflammatory bowel obstruction without fibrostenosis. It is difficult or sometimes impossible to distinguish inflammatory from fibrostenotic strictures by current diagnostic techniques, including imaging procedures and endoscopy, leaving the question of what is the gold standard for the differential diagnosis? Is there any method, which is superior to direct visualization by the surgeon and the pathologist as well as to patho-histological investigation? From the literature, good evidence cannot be retrieved. Thus, we based our evaluation of fibrostenosis on the assumption that surgical and pathological reports deliver the most reliable data on the presence of fibrostenosis and are, therefore, superior to imaging procedures in the diagnosis of fibrostenotic strictures on expert opinion.

aPCR is the most common known inherited thrombophilic disorder known until now^[16,17]. The genetic basis of aPCR is a single-point mutation in the gene encoding for coagulation factor V, also known as factor V Leiden. Factor V Leiden has a 2%-7% prevalence in most Caucasian populations and is identified in 20%-50% of patients with venous thromboembolism^[17,34-36].

In addition to the increased risk of venous thromboembolism, aPCR and other thrombotic risk factors have also been shown to accelerate the rate of fibrosis in inflammatory diseases, such as chronic viral hepatitis^[18,37,38]. Thrombotic obliterations of small portal and hepatic veins and activation of the stellate cells by

thrombin were assumed to cause increased fibrosis in the liver^[20]. The effect of thrombin is mediated through activation of thrombin receptors on the stellate cells, which are up-regulated during chronic liver injury^[39]. Furthermore, aPCR significantly increased pulmonary fibrosis in bleomycin-induced inflammatory lung injury in mice^[40]. Additionally, an association between aPCR and fibrosis has been described in a transgenic factor V Leiden mouse model with enhanced fibrin deposition in multiple tissues^[41]. In CD, an influence of the clotting system on the pathomechanism has been assumed based on the finding of intravascular fibrin deposition and capillary microthrombi^[21,22] and a reduced frequency of CD in patients with inherited haemophilic disorders^[23].

The association between aPCR or any other thrombotic risk factor and fibrosis in CD has not been previously investigated. An increased rate of fibrosis may lead to fibrostenotic lesions of the bowel, which often require surgery. Intestinal fibrosis and fibrostenosis are caused by uncontrolled proliferation of mesenchymal cells and excessive production of extracellular matrix proteins, including fibrillar collagen^[5,6]. Fibroblasts cultured from fibrostenotic lesions in CD patients, for instance, have been shown to synthesize an increased amount of collagen type III^[42]. A precondition for the development of fibrosis is a chronic transmural inflammation, which is a typical feature of CD. Infiltrating immune cells are considered to secrete cytokines and growth factors, such as transforming growth factor beta (TGF- β), in the mesenchymal layers, which might lead to activation of fibroblasts^[6,43].

Some risk factors predispose to fibrostenosis in CD. Several studies reported that CD-associated NOD2/CARD15 variants are associated with fibrostenotic lesions^[7-9] and, furthermore, that CD patients who are positive for ASCA, I2, anti-OmpC or pANCA are also more likely to develop fibrostenosis^[12-15]. Additionally, smoking has been shown to predispose to ileal localization of CD and to fibrostenotic lesions^[10-11]. But for all the itemized risk factors, it remains to be determined whether the association with fibrostenosis is independent or secondary to association with ileal involvement.

Immunosuppressive drugs do not seem to prevent the formation of fibrostenosis. Azathioprine has been shown to improve mucosal healing but not the development of fibrostenosis in CD, especially in the ileocecal region^[44-46]. A recent retrospective study on the use of immunosuppressive drugs and the need for intestinal surgery described only a tendency of reduced probability of formation of fibrostenosis over the time, despite a significant increase of the use of azathioprine in the same time period^[4]. However, the only immunosuppressive drug given to the patients in the present study was azathioprine, which was equally frequently administered to the patients of both groups. We can, therefore, exclude any influence of medical treatment on the results of our study.

Furthermore, there was no difference in smoking habits between the two patient groups. But all the other risk factors for fibrostenosis, such as NOD2/CARD15 variants, ASCA, I2, anti-OmpC, and pANCA, were not fully available and, therefore, not included in the analysis. It was thus not possible to investigate the influence of

these risk factors on our results. A further limitation might be the small number of patients included in the study. Since the effect of aPCR on fibrostenosis in CD was unknown prior to the present investigation, a calculation of the sample size was not possible. Our aim was to perform a pilot study on an association between aPCR and fibrostenosis in CD and by this way to generate the basis for a working hypothesis.

aPCR did not significantly influence the number of overall intestinal CD-related operations regardless of fibrostenosis. Therefore, we have no evidence that other indications for surgical treatment in CD except fibrostenotic strictures are influenced by aPCR.

In summary, our results give rise to this hypothesis that aPCR may be a risk factor for fibrostenosis in CD. The effect of aPCR might not be specific and coagulation might generally be involved in the development of fibrosis in CD, disposing patients with any thrombophilic disorder also to have an increased tendency to fibrostenosis. We are aware that our data should be verified by further larger trials, including data about other known risk factors for fibrostenosis. However, our study contains the first reference to an association between aPCR and fibrostenosis in CD and may provide a new insight in the pathogenesis of fibrostenosis in CD.

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RAPID COMMUNICATION

Xeroderma pigmentosum group D 751 polymorphism as a predictive factor in resected gastric cancer treated with chemo-radiotherapy

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Abstract

AIM: To evaluate the potential association of xeroderma pigmentosum group D (XPD) codon 751 variant with outcome after chemo-radiotherapy in patients with resected gastric cancer.

METHODS: We used PCR-RFLP to evaluate the genetic XPD *Lys751Gln* polymorphisms in 44 patients with stage III (48%) and IV (20%) gastric cancer treated with surgery following radiation therapy plus 5-fluorouracil/leucovorin based chemotherapy.

RESULTS: Statistical analysis showed that 75% (12 of 16) of relapse patients showed *Lys/Lys* genotype more frequently ($P = 0.042$). The *Lys* polymorphism was an independent predictor of high-risk relapse-free survival from Cox analysis (HR: 3.07, 95% CI: 1.07-8.78, $P = 0.036$) and Kaplan-Meier test ($P = 0.027$, log-rank test).

CONCLUSION: XPD *Lys751Gln* polymorphism may be an important marker in the prediction of clinical outcome to chemo-radiotherapy in resected gastric cancer patients.

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Key words: Xeroderma pigmentosum group D gene; Polymorphism; Gastric cancer; Radiotherapy

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INTRODUCTION

The xeroderma pigmentosum group D (XPD) gene encodes a protein required for nucleotide excision repair (NER). This product recognizes and repairs a wide range of structurally unrelated lesions such as bulky adducts caused by UV light, environmental agents, cross links and oxidative damage^[1,2]. Moreover, XPD gene is one of the components of basal transcription factor IIIH (TFIIH), participating also in transcription initiation. Since XPD is involved in both transcription and NER, it may be able to repair other types of damage, such as radiation therapy-induced damage. Because XPD interacts with many different proteins as part of TFIIH transcription factor, amino acid variants in different domains of XPD, such as 683 and 751, may affect different protein interactions, and result in expression of different phenotypes^[3,4].

Intrinsic and acquired resistance to cancer chemotherapeutic agents results from polymorphisms in genes encoding DNA repair enzymes. Differences in responsiveness of cancer cells to anticancer agents can be differently affected by changes in repair efficiency. Clinical outcomes after chemotherapy may be influenced by pharmacogenetic polymorphisms in DNA repair enzymes. In this sense, three single nucleotide polymorphisms (SNP) in the XPD gene (in codons 156, 312, and 751) are related with different DNA repair capacity^[5-7]. Thus, variants generated by amino acids can change *Asp312Asn* (exon 10, G > A substitution) and *Lys751Gln* (exon 23 A > C substitution) located in conserved regions of the XPD protein, and are associated with lower efficiency of damage repair and significantly higher background frequency of apoptotic cells in irradiated lymphocytes^[8].

Although gastrectomy is the only potentially curative treatment in gastric-cancer patients, the overall survival results remain unsatisfactory. The main factor accounting for high mortality is relapse after surgical resection. During the past few decades, the principle of combined treatment modality has been developed and applied in various solid tumors including gastric cancer. In order to prevent recurrence and increase the cure rate of gastric-cancer after surgery, multiple studies using variable treatment modali-

ties have been undertaken. One of the landmark studies reported that chemo-radiotherapy can significantly improve survival after resection of stage IB to stage IV gastric cancers^[9]. Chemo-radiotherapy has been increasingly recognized as a standard of care since then^[10,11]. However, whether adjuvant chemo-radiotherapy can prolong the survival of patients with extensive lymph node dissection remains debatable. In this paper, we evaluated the potential role of XPD codon 751 variant in the outcome of 44 patients with resected gastric cancer after chemo-radiotherapy.

MATERIALS AND METHODS

Patients

Characteristics of the patients are listed in Table 1. From October 1992 to January 1999, 44 patients consisting of 32 men (73%) and 12 women (27%) with a median age of 60 years (range: 33-77 years) with diagnosis of gastric cancer after having undergone gastrectomy were treated with radiation plus 5-fluorouracil (5-FU) and leucovorin. Thirty-two percent of the patients had stage I - II and 68% stage III-IV gastric cancer at the time of diagnosis (Table 1). The median follow-up time was 46.9 mo (range: 5.33 to 124.06 mo). The median time to progression was 37.5 mo (range: 2.3 to 95.4 mo). This study was conducted in Navarra Hospital Center, and informed consent was obtained from all the patients for using their tissues. The clinicopathological information of each subject was obtained from the tumor registry at Navarra Hospital.

Treatment schedule

The patients received postoperative treatment with 5-FU plus leucovorin and local radiation 20-40 d after gastrectomy. This chemotherapy regimen developed by the North Central Cancer Treatment Group^[12] was used before and after radiation. Chemotherapy (fluorouracil, 425 mg/m² per day, and leucovorin, 20 mg/m² per day) was initiated on d 1 followed by chemo-radiotherapy on d 28 after the initial cycle of chemotherapy. Chemo-radiotherapy consisted of 4500 cGy of radiation at 180 cGy/d, 5 d/wk for 5 wk, with 5-FU (400 mg/m² per day) and leucovorin (20 mg/m² per day) on the first four and the last three days of radiotherapy. One month after radiotherapy, two 5-d cycles of 5-FU (425 mg/m² per day) plus leucovorin (20 mg/m² per day) were given every other month.

The 4500 cGy of radiation was delivered in 25 fractions (5 d/wk) to the tumor bed, regional nodes and 2 cm beyond the proximal and distal margins of resection. The adjuvant treatment was performed as previously described^[9].

Samples and DNA extraction

Surgical specimens (paraffin blocks) were cut into 5 µm-thick sections for molecular analysis.

Three tissue sections were transferred into a micro centrifuge tube and 1.2 mL of xylene was added. After centrifugation at 14 000 r/min for 5 min at room temperature (RT), the supernatant was removed. Subsequently, the tissue samples were washed in 1.2 mL of 96 mL/L

Table 1 Characteristics of the patients

Baseline factors	n (%)
Sex	
Male	32 (73)
Female	12 (27)
Age (yr)	
≤ 50	16 (36)
51-64	13 (30)
≥ 65	15 (34)
Median (Range)	60 (33-77)
Stage at diagnostic	
I - II	14 (32)
III A + III B	21 (48)
IV	9 (20)
Histotype	
Intestinal	9 (20)
Diffuse	35 (80)
Grading	
I	3 (7)
II	8 (18)
III	33 (75)
Tumor location	
Cardia	9 (21)
Fundus	9 (21)
Body	10 (24)
Antrum	14 (33)
Gastrectomy	
R0	35 (80)
R1	8 (18)
R2	1 (2)
XPD 751	
Lys/Lys	19 (51)
Lys/Gln	14 (38)
Gln/Gln	4 (11)
Unknown	7

ethanol. After centrifugation at 14 000 r/min for 5 min at RT, the supernatant was discarded. The washing procedure was repeated another time. The samples were dried for 3-5 min, in a vacuum pump (any letter between were and dried).

DNA was extracted using the QIAamp DNA Mini Kit (Qiagen-IZASA- Barcelona Spain). The deparaffinized samples were re-suspended in 180 µL of ATL buffer plus 20 µL of proteinase K and incubated overnight at 56°C. The samples were processed according to the provided protocol. The purified DNA was finally eluted in a total volume of 150 µL. DNA yield was quantified by NanoDrop 3.0.0 (Nucliber, Wilmington, Delaware USA).

About 7-509 mg/L of DNA was extracted and 45-200 ng of genomic DNA was used as template. PCR/RFLP-based assays were performed as described previously^[5] with slight modifications. The PCR product (20 µL) was digested with 25 units of Pst I enzyme (New England BioLabs, -IZASA, S.A, Barcelona-, Spain) in 50 µL reaction mixture for 1 h. The digestion product was visualized in 3% agarose gel (Pronadisa, Madrid, Spain). The wild-type homozygote was defined by 104- and 220-bp banding patterns; the heterozygote by 63-, 104-, 157-, and 220-bp fragments; and variant homozygote

Table 2 Toxicity evaluated

Toxicity grade III-IV	n (%)
No	28 (64)
Yes	16 (36)
Toxicity type	
Hematological	10 (62)
Digestive	3 (19)
Others	3 (19)

Table 3 Relapse site

Relapse	n (%)
No	24 (54.5)
Yes	20 (45.5)
Relapse sites	
Local	5 (25)
Distant	10 (50)
Local + Distant	5 (25)

by 63-, 104-, and 157-bp fragments. Some samples were noted as “unknown” because they could not be amplified due to a relatively frequent occurrence of PCR inhibitory substances in samples prepared with this DNA extraction method (Table 1). Furthermore, three DNA samples corresponding to each genotype selected by direct sequencing were used. The results were in concordance with RFLP genotyping.

Statistical analysis

This study was designed to analyze the role of XPD polymorphism in the prediction of relapse. Relapse free-survival (RFS) was defined as the time from the start of chemotherapy to the first evidence of disease progression. RFS was calculated using the Kaplan-Meier method. Contingency tables and chi-square test (χ^2) were used to summarize the association of relapse with XPD polymorphism. All *P* values were two-sided. Cox proportional hazards models were also used to evaluate the different variables considered. All statistical tests were conducted by SPSS software 11.0 version for Windows (SPSS, Inc. Chicago). *P* ≤ 0.05 was considered statistically significant.

RESULTS

Thirty-seven patients were evaluated for the *Lys751Gln* polymorphism. Seven patient were not assessed due to poor sample extraction quality. Fifty-one percent (19 of 37) of the patients were homozygous for the *Lys/Lys* genotype, 38% (14/37) heterozygous for *Lys/Gln*, and 11% (4 of 37) homozygous for the glutamine variant (Table 1). The *751Gln* allele frequency was 0.33, similar to that observed in other studies^[5,7].

Sixteen patients (36%) developed grade III-IV toxicity. Hematological toxicity was found in 62% (10/16) patients compared with gastrointestinal and other toxicity in 19% (3/16) patients (Table 2). However, there was no

Table 4 Results of chi-square test for disease relapse

Relapse	XPD			Stage		
	<i>Lys</i>	<i>Lys/Gln</i>	<i>Gln/Gln</i>	I + II	III A + III B + IV	
No	7	11	3	11	13	
Yes	12	3	1	3	17	
<i>P</i>		0.042			0.050	

Table 5 Unadjusted XPD genotype and relapse-free survival (RFS)

XPD	RFS (median)	Hazard Ratio	95% CI
<i>Lys/Lys</i>	11.79	1.0	Reference
<i>Lys/Gln</i>	14.33	0.35	0.11 to 1.09
<i>Gln/Gln</i>	23.33	0.25	0.03 to 1.96

Table 6 Adjusted Cox multivariate analysis for RFS

XPD	RFS (median)	Hazard ratio	95% CI	<i>P</i>
<i>Lys/Lys</i>	11.79	3.07	1.07 to 8.78	0.036
<i>Lys/Gln</i> + <i>Gln/Gln</i>	20.26	1.0	Reference	

association between XPD genotype and toxicity (data not shown).

Gastrectomy with D1 lymphadenectomy was performed in all patients. Of the 44 patients, 20 (45.5%) had relapse and 17 (85%) of them died. The first relapse site was local in 5, distant in 10, and both local and distant in 5 (Table 3).

Statistical analysis showed that 75% (12 of 16) of relapse patients showed *Lys/Lys* genotype more frequently (*P* = 0.042, Table 4).

Other clinico-pathological features such as histology, grading and localization were not significant (data not shown).

The relative risk of progression (with *Lys/Lys* genotype used as the reference) was 0.35 (95% CI: 0.11-1.09) for patients carrying the *Lys/Gln* genotype and 0.25 (95% CI: 0.03-1.96) for the *Gln/Gln* group (Table 5).

Only XPD genotype and stage to diagnosis showed a significant relation with cancer relapse and were evaluated as potential predictors of RFS by Cox test. We used the combined genotypes *LysGln/GlnGln* as reference in these analyses and found that the *Lys/Lys* genotype was more strongly associated with disease progression (*P* = 0.036) than the combined *LysGln/GlnGln* genotype (adjusted HR: 3.07, 95% CI: 1.07-8.78) (Table 6, Figure 1).

DISCUSSION

Most cases of gastric cancer are diagnosed at an advanced stage with poor prognosis. Surgery remains the only potentially curative treatment, but it is associated with a high rate of local recurrence and distant metastases. When irradiation is combined with surgical resection for all or a

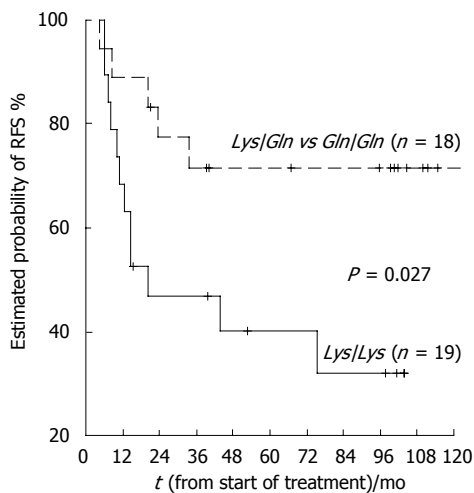


Figure 1 XPD751 polymorphism and RFS under radio-5Fu/Lv treatment.

majority of tumors, both survival and local control appear to be better than those for the unresected patients^[13]. Epidemiological studies have shown that *Gln* variant at XPD-751 polymorphism is partially able to repair DNA in lung cancer^[7,14]. In this sense, cancer treatment is to cause DNA damage and tumor cell apoptosis. *Gln* variant seems to show a lower ability to repair DNA damage and cancer patients are more sensitive to chemo-radiotherapy. In contrast, the presence of allele *Lys* represents a positive effect in different DNA repair pathways and a possible poor response to cancer treatment. In this study, we found a significant relationship between clinical response to chemo-radiotherapy and the XPD *Lys751Gln* polymorphism. Patients with the *Lys/Lys* genotype were more likely to have relapse compared to those with the combined *Lys/Gln* and *Gln/Gln* genotype.

Scientific publications are available but with diverging results in the XPD751 polymorphism and cancer risk or treatment efficiency^[3,5-7]. Thus, decreased DNA repair has been associated with *Lys* allele^[6]. Epidemiological study has reported association of this allele with a higher risk of basal cell carcinoma^[5] even though no significant relationship between this polymorphic gene and DNA repair proficiency has been reported^[15,16]. Spitz *et al*^[7] and other research group^[17] reported a suboptimal DNA repair capacity (DRC) particularly in subjects who were homozygous for 751*Gln* alleles. Moreover, a recently study has found a tendency toward a higher background frequency of apoptotic cells in irradiated lymphocytes carrying variant homozygote *Gln* at XPD codon 751^[8]. Our results are in agreement with these studies, suggesting that heterozygote and homozygote *Gln* alleles may therefore increase their clinical response to suboptimal DNA damage repair after treatment.

Repair of DNA damage is a complex process^[1,18,19]. Functional protein of XPD could be affected by a structural change (*Lys* → *Gln*) from a basic to a polar amino acid change located at about 50-base upstream from the poly (A) signal^[5]. Moreover, close proximity in the genome between XPD and other polymorphic DNA repair genes such as X-ray repair cross complementing (XRCC1) and excision repair cross complementing (ERCC1) may influ-

ence the interaction between them leading to different or synergic DNA repair capability. A recent molecular epidemiology study has genotyped 44 SNPs in 20 genes involved in four DNA damage repair pathways, showing that homozygous *Gln751Gln* and other gene variants genotyped are highly associated with lung cancer risk^[20]. In the same way, variant *Gln* allele and some nucleotide and base excision repair (NER and BER, respectively) variant gene members are associated with increased level of polycyclic aromatic hydrocarbon-DNA adducts^[15,21].

In conclusion, XPD *Lys751Gln* polymorphism may be an important marker of genotoxicity that can predict the clinical outcome of resected gastric cancer treated with chemo-radiotherapy.

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Perinatal events and the risk of developing primary sclerosing cholangitis

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explained, so our findings do not strongly support the hypothesis of a significant role of perinatal events as a risk for the development of PSC later in life.

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Key words: Inflammatory bowel disease; Perinatal factors; Sclerosing cholangitis

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Abstract

AIM: To investigate whether perinatal events, intrauterine or postpartum, are associated with the development of primary sclerosing cholangitis (PSC) later in life.

METHODS: Birth records from 97 patients with adult PSC in Sweden were reviewed. Information on perinatal events including medications and complications during pregnancy, gestation length, birth weight and length were collected. Two control children of the same sex were selected for each subject. Conditional multiple logistic regression was used to assess associations of the perinatal measures with development of PSC.

RESULTS: No significant associations were found between gestational age, birth length, breastfeeding, and the majority of medical complications including infections or medication during pregnancy for the mothers or postpartum for the children. Vaginal bleeding and peripheral oedema showed associations with PSC, with matched odds ratios of 5.70 (95% CI, 1.13-28.83) and 2.28 (95% CI, 1.04-5.03), respectively.

CONCLUSION: The associations of vaginal bleeding and oedema with subsequent PSC cannot readily be

INTRODUCTION

Primary sclerosing cholangitis (PSC) is a complex disease likely to involve multiple susceptibility genes, environmental and immunological risk factors. PSC can present at any age and is characterised by a long subclinical asymptomatic period in many cases^[1]. The aetiology of the disease is unknown. PSC is closely associated with inflammatory bowel disease (IBD) most commonly ulcerative colitis (UC)^[1,2]. A role for perinatal events in the aetiology of IBD has been suggested. Non-specific exposures such as infections and more specific factors including measles virus infection and vaccination as well as appendectomy have been shown to be associated with later development of IBD^[3-6]. There is evidence of genetic susceptibility in both IBD and PSC^[7,8] and there is a possibility that some immune profiles inherited by the offspring may increase the risk for both these autoimmune diseases as well as for perinatal events and other complications during pregnancy. Since perinatal events represent an increased risk for IBD^[6], some early life events may be a direct risk for PSC. The close association between IBD and PSC has been suggested to be part of the pathogenetic explanation for PSC through the existence of an entero-hepatic circulation of lymphocytes. Some lymphocytes generated in the gut during active IBD may subsequently persist as long-lived memory cells and be activated in the liver resulting in hepatic inflammation and the development of PSC. The molecular basis for this hypothesis is that liver and gut share the same lymphocyte homing receptors^[9]. However,

this hypothesis does not explain why only 5% of patients with IBD eventually develop PSC^[10] and why PSC can precede IBD. Whether perinatal factors play a role for the development of PSC later in life has not been previously evaluated.

The aim of this study was to assess whether perinatal events, intrauterine or postpartum, are associated with an increased risk of PSC.

MATERIALS AND METHODS

All patients with PSC treated at the five largest University Hospitals in Sweden between 1970 and 1998 were selected for this study ($n = 311$). Through the unique national registration number assigned to every Swedish citizen we were able to trace birth parish to identify the hospital where delivery occurred. To trace the medical records associated with delivery and pregnancy from these patients, we searched the respective hospital archives. The detailed medical records from 97 patients with PSC were collected. Individuals born abroad or delivered at home by midwives were excluded. As controls, we selected two children of the same sex as the subject, who were delivered at the same hospital immediately after the subjects. The controls had to be alive and living in Sweden at the date of PSC diagnosis for the case. Controls who had died or emigrated before the diagnosis of their matched case were excluded. One hundred and seventy five controls remained for analysis.

The diagnosis of PSC was based on biochemical, histological and cholangiographic features^[11]. Onset of PSC was defined as first cholangiogram consistent with the diagnosis. Adult PSC was defined as onset of PSC at 16 years of age or later. A diagnosis of UC was based on a typical history and characteristic endoscopic and histological findings^[12]. All 97 patients with PSC had undergone colonoscopy to look for IBD and no endoscopic or histological signs of IBD were found in the non-IBD PSC patients.

For both, subjects and controls, we retrieved information on the age of parents, delivery method, parity and maternal complications including proteinuria, peripheral oedema, hypertension or other diseases during the pregnancy. Other conditions and factors during pregnancy included surgeries, infections, nausea, anemia, varices, constipation, thrombosis or any other disease reported by the patients to the midwife and documented in the patient's record. Information concerning medication during pregnancy, gestational age, birth height and weight, weight of the placenta and medical problems experienced by the mother or child postpartum were also collected. Small and large for gestational age were defined as birth weights two standard deviations below and above normal for the gestational age. All data from the birth records were personally collected by one researcher (U.L). The study was approved by the Ethics committee at Karolinska Institute, Karolinska University Hospital, Huddinge, Sweden.

Statistical analysis

Mean values of continuous measurements were compared

Table 1 Description of perinatal factors in 97 PSC patients and 175 controls

	Patients with PSC ($n = 97$)	Controls ($n = 175$)	<i>P</i>
Age of the mother, yr (mean \pm SD)	27.8 \pm 5.1	27.4 \pm 6.2	NS
Hospital stay days (mean \pm SD)	7.9 \pm 3.2	8.0 \pm 4.7	NS
Birth weight in grams (mean \pm SD)	3501 \pm 537	3557 \pm 480	NS
Birth length in cm (mean \pm SD)	51.0 \pm 2.2	51.0 \pm 1.9	NS
Ponderal index (Weight/height ³)	2.6 \pm 0.3	2.7 \pm 0.3	NS
Vaginal delivery, n (%)	90 (93%)	166 (95%)	NS
Jaundice post partum, n (%)	7 (7%)	14 (8%)	NS
Breastfeeding at discharge from hospital, n (%) ¹	94 (97%)	170 (97%)	NS

¹Missing data in 2 patients.

in order to describe the characteristics of the case and control groups. To assess associations of the perinatal measures with PSC, conditional multiple logistic regression was used. All investigated parameters were modelled as a series of binary dummy variables. After investigation of univariate relationships, all measures associated with PSC ($P < 0.05$) that were statistically significant were included in multivariate analysis using conditional logistic regression. The final conditional logistic regression model excluded redundant measures co-linear with other significant risk factors.

RESULTS

The mean age at PSC diagnosis in the 97 patients was 40 \pm 12 years (\pm SD). 78% (76/97) were men, 80% (78/97) had a concomitant diagnosis of IBD. Sixty eight patients had UC, nine were diagnosed with Crohn's disease and one patient had indeterminate colitis.

A description of maternal and perinatal factors in the 97 PSC cases and the 175 controls is given in Table 1. Birth weight (BWT) was divided into five equally sized groups, where BWT 1 represented the lowest birth weight. Compared with the middle category, only the second lowest birth weight group was associated with PSC in a statistically significant manner. There were no statistically significant differences between the rates of maternal infections in subjects and controls. During pregnancy, maternal infections were reported in four cases (pneumonia ($n = 1$), tuberculosis ($n = 1$), urinary tract infection ($n = 1$), rubella in the first trimester ($n = 1$)). The child born to the rubella-infected mother was female and developed Crohn's disease before PSC was diagnosed. In the control group, one mother suffered from tuberculosis. None of the mothers were diagnosed with IBD or chronic liver disease. 9.3% of the PSC cases and 8.0% of the controls had postpartum medical issues. The most common problems observed in the children were skin lesions and asphyxia-related problems, in both groups. The summated frequency of

Table 2 Matched odds ratios and 95% confidence intervals for the maternal perinatal risk factors studied in 97 patients with PSC and 175 controls

Risk factor	¹ n with PSC (%)	n without PSC(%)	OR (95% CI)	P
Vaginal bleeding	6 (6.2)	2 (1.1)	5.70 (1.13-28.83)	0.035
Oedema	15 (15.5)	13 (7.4)	2.28 (1.04-5.03)	0.040
Eclampsia	0	1 (0.6)	a	
Albuminuria during pregnancy	10 (11.4)	8 (5.2)	2.33 (0.88-6.15)	0.087
Albuminuria at arrival at the hospital	16 (17.2)	20 (13.1)	1.35 (0.66-2.76)	0.416
Albuminuria postpartum	13 (13.7)	22 (13.3)	1.02 (0.48-2.14)	0.963
Medical problems postpartum-mother	11 (11.3)	22 (12.6)	0.89 (0.41-1.93)	0.765
Medical problems postpartum-child	9 (9.3)	14 (8.0)	1.18 (0.49-2.83)	0.717
Breastfeeding	95 (97.9)	166 (97.1)	1.44 (0.27-7.67)	0.668
BWT 1	23 (23.7)	31 (17.7)	1.44 (0.57-3.65)	0.437
BWT 2	26 (26.8)	28 (16.0)	2.89 (1.17-7.18)	0.022
BWT 3	16 (16.5)	39 (22.3)	Reference	
BWT 4	14 (14.4)	42 (24.0)	0.76 (0.29-2.01)	0.576
BWT 5	18 (18.6)	35 (20.0)	1.44 (0.57-3.63)	0.437
Gestational weeks < 38	8 (8.4)	7 (4.1)	2.20 (0.77-6.29)	0.144
Gestational weeks 38-42	78 (82.1)	150 (87.2)	Reference	
Gestational weeks > 42	9 (9.5)	15 (8.7)	1.16 (0.48-2.76)	0.755
Small for gestational age			1.09 (0.25-4.71)	
Large for gestational age			2.33 (0.61-8.91)	

BWT 1-5, 5ths; 1, lowest ; BWT, body weight. ¹The cases are matched with controls for sex, age and hospital ^aNo estimate due to an empty cell.

all postpartum medical problems for mothers of PSC patients was 11.3% and 12.6% for the controls, dominated by bleeding-anaemia in both groups. This difference is not statistically significant.

The matched odds ratios for perinatal factors are shown in Table 2. Both vaginal bleeding and peripheral oedema are statistically significantly associated with PSC. There was also an association of albuminuria during pregnancy with PSC that is not statistically significant. There was no association between jaundice and the risk of PSC. The non-matched odds ratios did not differ notably from the matched odds ratios shown in Table 2 (data not shown).

A multivariate analysis was conducted for the factors associated with a statistically significant increased risk of PSC: vaginal bleeding, peripheral oedema and birth weight. For vaginal bleeding, the adjusted OR (95% CI) is 6.7 (1.3-34.8), 2.4 (1.1-5.3) for peripheral oedema and 2.24 (1.00-5.02) for the second lowest birth weight category. An additional adjustment for gestational age was conducted separately (data not shown) and did not significantly alter the odds ratios for birth weight, vaginal bleeding or peripheral oedema.

DISCUSSION

To our knowledge, this is the first study investigating the associations of perinatal factors with PSC. The rationale for conducting such a study is that the foetal environment may be a contributing factor in the aetiology of some adult conditions such as diabetes, insulin resistance and rheumatoid arthritis^[13,14]. In addition, IBD (both CD and UC) is closely associated with PSC and “non-infectious

health events” during pregnancy^[4,6]. In celiac disease, which is also associated with PSC^[15], it has been shown that a low birth weight for gestational age and neonatal infections are associated with later development of celiac disease^[16].

We found no associations between PSC and birth length, gestational age, or medical problems for the mother or child, during pregnancy or postpartum. Mothers who had specific infections were identified. Neither individually nor combined were the specific infections associated with PSC risk. However, the number of events is low so detailed analysis was not possible. The association of PSC with birth weight was limited to one part of the birth weight distribution and there was no evidence of a trend. This suggests a chance association that should not be over-interpreted.

Some specific factors, vaginal bleeding during pregnancy and peripheral oedema, proved to be statistically significant in association with higher PSC risk. Interpretation of these data should be cautious. The number of women with vaginal bleeding was small and a clear mechanism to link this event with PSC is not readily apparent. As more women displayed peripheral oedema, its association with PSC is of greater relevance, yet it is a somewhat non-specific symptom, and again, a biologically plausible mechanism linking it with PSC is not obvious. It may be worthwhile to note that peripheral oedema is a symptom of pre-eclampsia; the other important symptoms are hypertension and proteinuria. Only one mother in this study was diagnosed with pre-eclampsia and we could not fully evaluate possible sub-clinical diseases as data were missing for proteinuria in both groups. However, the weak association of proteinuria with the risk for PSC provides further, but limited, evidence that some symptoms of pre-

eclampsia are associated with PSC. If the link between PSC and these maternal symptoms is not due to chance, then they could indicate some inherited characteristics that represent susceptibility to PSC. Alternatively, they might represent foetal exposure, possibly to pro-inflammatory factors, which increase the risk of PSC among susceptible individuals, perhaps through the initiation of an autoimmune process.

Although the detailed aetiology of PSC is unknown, its close association with IBD indicates that these two diseases share important risk factors relevant to exposure or susceptibility.

It is possible that risk factors identified by this study could be intermediates in a causal pathway between IBD and PSC: this could not be investigated here due to the small number of IBD-free subjects. However, it is also possible that the associations are specific to PSC or identify a subset of people with both diagnoses. IBD may indicate greater susceptibility to PSC, but exposure to other risk factors may be required to initiate the pathogenesis of this disease. Given the close association of PSC and IBD, known risks for IBD should be considered.

Bacterial colonisation of the gut is implicated in the aetiology of IBD; this occurs first in early life and the critical stages of early gut colonisation include exposure to bacteria in the birth canal, maternal faecal bacteria and during weaning^[17]. Breastfeeding is important for colonic bacterial colonisation^[17]. Previous studies investigating the association between breast feeding and development of IBD have shown inconsistent results^[18,19]. In the present study, there was a similarly high frequency of breastfeeding among patients and controls (97% in both groups). However, the observation period was short (approximately one week) since breastfeeding was only registered at the time when the mothers were discharged from the hospital and no follow-up was available.

Although the number of subjects included in this study was not large, it includes a high proportion of Swedish PSC patients between the years 1970 and 1998. Differential selection bias is not a concern as subjects and controls were closely matched and the analysis was conditional, such that patients were compared with their matched controls. The controls were selected from the same birth unit (next two consecutive births with the same gender as the subject) and the data was retrieved by the same person. Furthermore, differential information bias is not a determining factor either, as the selection of cases or controls was not influenced by whether perinatal adverse events occurred or not. The general characteristics of the PSC patients in the present study, such as age at onset of PSC, association with IBD and sex distribution, are similar to other studies, suggesting that selection bias among cases is unlikely^[1,2].

In summary, no significant associations with PSC were found for gestational age, birth length, breastfeeding, or most medical complications, including infections, during pregnancy for the mothers or postpartum for the children. The associations with vaginal bleeding and some symptoms of pre-eclampsia could be due to chance,

but should be considered as putative risk factors by further studies. Overall, our findings do not support the hypothesis of a substantial role for perinatal events in the aetiology of PSC later in life.

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Loss of disabled-2 expression is an early event in esophageal squamous tumorigenesis

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silencing is only one of the mechanisms causing loss of DAB2 expression in ESCCs.

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Key words: *Disabled-2*; DOC-2; Esophageal cancer; Promoter hypermethylation; Dysplasia

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Abstract

AIM: *Disabled-2 (DAB2)* is a candidate tumor-suppressor gene identified in ovarian cancer that negatively influences mitogenic signal transduction of growth factors and blocks ras activity. In a recent study, we observed down-regulation of *DAB2* transcripts in ESCCs using cDNA microarrays. In the present study, we aimed to determine the clinical significance of loss of *DAB2* protein in esophageal tumorigenesis, hypothesizing that *DAB2* promoter hypermethylation-mediated gene silencing may account for loss of the protein.

METHODS: *DAB2* expression was analyzed by immunohistochemistry in 50 primary esophageal squamous cell carcinomas (ESCCs), 30 distinct hyperplasia, 15 dysplasia and 10 non-malignant esophageal tissues. To determine whether promoter hypermethylation contributes to loss of *DAB2* expression in ESCCs, methylation status of *DAB2* promoter was analyzed in *DAB2* immuno-negative tumors using methylation-specific PCR.

RESULTS: Loss of *DAB2* protein was observed in 5/30 (17%) hyperplasia, 10/15 (67%) dysplasia and 34/50 (68%) ESCCs. Significant loss of *DAB2* protein was observed from esophageal normal mucosa to hyperplasia, dysplasia and invasive cancer ($P_{\text{trend}} < 0.001$). Promoter hypermethylation of *DAB2* was observed in 2 of 10 (20%) *DAB2* immuno-negative ESCCs.

CONCLUSION: Loss of *DAB2* protein expression occurs in early pre-neoplastic stages of development of esophageal cancer and is sustained down the tumorigenic pathway. Infrequent *DAB2* promoter methylation in ESCCs suggests that epigenetic gene

INTRODUCTION

Esophageal cancer is the most aggressive gastrointestinal malignancy ranking as the 6th most common cancer among males and 9th most common cancer among females globally^[1]. Squamous cell carcinoma is the predominant histological subtype of esophageal cancer, characterized by high mortality rate and strong association with dietary habits and life style in India^[2-4]. It is the 2nd most common cancer among males and 4th most common cancer among females in India^[5]. Despite advances in multimodality therapy, due to late stage of diagnosis and poor efficacy of treatment, the prognosis for patients with ESCC still remains poor with an average 5-year survival of < 10% globally^[6,7] and < 12% in India^[8,9].

DOC-2/*DAB2* (differentially expressed in ovarian carcinoma- 2/disabled-2) is a putative tumor suppressor gene that encodes a 96-ku mitogen-responsive phosphoprotein involved in signal transduction^[10-15]. It inhibits mitogenic stimulation via the Ras pathway by binding to Grb2^[13,14]. *DAB2* has been shown to act as a negative regulator of c-Src in normal prostatic epithelium and cancer^[16]. This interaction causes inactivation of Erk and Akt proteins critical for proliferation and survival of prostate cancer cells^[16,17]. *DAB2* is found in association with transforming growth factor- β (TGF- β) type I and II receptors, while directly binding to the TGF- β signaling intermediates Smad2 and Smad3 through the PID domain^[18]. *DAB2* plays an important regulatory role in cellular differentiation and induction of differentiation in the absence of *DAB2* expression commits the cell to apoptosis^[19]. *DAB2* functions as a negative regulator of canonical Wnt signaling by stabilizing the beta-catenin

degradation complex^[20]. Recently, treatment of mouse F9 embryonic carcinoma cells with glycosylceramide synthase inhibitors has been shown to result in depletion of gangliosides and delayed expression of DAB2, suggesting their involvement in F9 cell differentiation^[21]. The aberrant expression of DAB2 has been reported in tumors, such as ovarian, prostate, choriocarcinoma, and breast^[11,22-26]. Histologically, elevated levels of DAB2 are associated with an enriched basal cell compartment, a progenitor cell for glandular epithelium and may be involved in the homeostasis of rat prostate regeneration^[23]. In addition, stable expression of DAB2 in cancer cell line has been shown to significantly reduce its *in vitro* growth rate, concomitant with an increase in cells in G₁ and decrease in anchorage-independent growth on soft agar^[11,22,23]. Therefore, DAB2 appears to be a potent negative regulator of cancer cell growth.

In a recent study, we observed down-regulation of *DAB2* transcripts in ESCCs using cDNA microarrays (data not shown). To our knowledge, the clinical significance of down-regulation of DAB2 in ESCC remains to be determined. In the present study, we analyzed DAB2 protein expression in different stages of development of esophageal cancer viz., primary ESCC and paired non-malignant normal, hyperplasia and dysplasia. Loss of DAB2 protein was observed in high proportion of ESCCs and dysplasia. Therefore, we hypothesized epigenetic silencing of *DAB2* gene in ESCCs. To test this hypothesis, the methylation status of the putative promoter (exon 1) of *DAB2* was analyzed using methylation-specific PCR in ESCC tissues that showed loss of DAB2 protein.

MATERIALS AND METHODS

Tissue samples

The study was approved by Institutional Human Ethics Committee and informed consent was obtained from the patients prior to enrolment in the study. The tissue samples used in this study were collected from Department of Gastrointestinal Surgery, All India Institute of Medical Sciences, New Delhi, India. All the samples were histologically confirmed to be either ESCCs, esophageal hyperplasia, dysplasia or non-malignant tissues by the pathologist (SDG). The samples included 50 histologically confirmed ESCCs, 10 non-malignant esophageal mucosa, 30 hyperplasia and 15 dysplasia.

Immunohistochemistry

Immunohistochemical analysis of DAB2 protein was carried out in paraffin-embedded tissue sections (5 µm thickness). Briefly, the sections were deparaffinized in xylene, hydrated and incubated with 30 mL/L H₂O₂ in methanol for 5 min to inactivate the endogenous peroxidase. Slides were washed with Tris-buffered saline (TBS, 0.1 mol/L, pH7.4) and heated for 15 min at 100°C in 10 mmol/L sodium citrate buffer (pH 6.0). Thereafter, sections were incubated with anti-disabled-2 goat polyclonal antibody (C-20, dilution 1:50, Santa Cruz Biotechnology Inc., Santacruz, CA) at 4°C overnight in humidified chamber. Sections were incubated with biotinylated anti-mouse antiserum with horseradish

peroxidase streptavidin conjugate (DAKO Labs, Glostrup, Denmark). After every incubation step, slides were washed with TBS thrice and color was developed using 3,3'-diaminobenzidine hydrochloride (DAB). Sections were counterstained with Mayer's hematoxylin, and mounted with DPX mountant for evaluation. Normal ovary tissue sections were taken as positive control for DAB2 and in the negative control primary antibody was replaced by isotype-specific IgG (data not shown).

Bisulfide modification

Genomic DNA from tissues was isolated by phenol-chloroform method. Genomic DNA was treated with sodium bisulfite (Sigma-Aldrich, Bangalore, India) as previously described^[27]. Briefly, 1 µg of DNA was denatured with 0.2 mol/L NaOH for 10 min at 37°C. Thirty microliters of 10 mmol/L hydroquinone (Sigma Aldrich) and 520 µL of 3 mol/L sodium bisulfite (pH 5.0) were added, followed by incubation at 50°C for 16 h. The modified DNA was purified using Wizard DNA purification columns (Promega, Madison, WI). The purified DNA was desulphonated with NaOH and precipitated with absolute ethanol in the presence of glycogen and ammonium acetate. DNA was resuspended in 20 µL of 1 mmol/L Tris (pH 8.0) and used for PCR amplification.

Methylation-specific PCR

Bisulfite-treated genomic DNA was amplified with either a methylation-specific or unmethylation-specific primer set for 35 cycles at 95°C for 5 min (hot started by adding Taq polymerase), followed by cycling with denaturation at 95°C for 30 s, primers annealing at 60°C for 30 s, and extension at 72°C for 1 min, as well as a final extension step at 72°C for 5 min. Methylation-specific primers span 6 CpG dinucleotides numbered 19-21 (forward) and 35-37 (reverse) of *DAB2* exon1 (Gene accession No. AF218839). Similarly, unmethylation-specific primers span 8 CpG dinucleotides numbered 19-22 (forward) and 35-37 (reverse). The methylation-specific primers were designed using 5'-TATTTTTCGTCGGGAGTGGTCGC-3' as the forward primer and 5'-ACTAACTATTACCTCCGTAAA ACG-3' as the reverse primer. The unmethylation-specific primers for site S1 were designed using 5'-GAATTATATT TTTTGTGGGAGTGGTTGT-3' as the forward primer and 5'-CCAACTAACTATTACCTCCATAAAACA -3' as the reverse. These primer sequences were reported by Akiyama *et al*^[28].

RESULTS

Immunohistochemical analysis of DAB2

Immunohistochemical analysis was carried out to determine the expression of DAB2 protein in different stages of esophageal tumorigenesis. Table 1 summarizes the clinicopathological parameters of ESCC patients and expression status of DAB2 protein in the tumors. Strong cytoplasmic staining of DAB2 was observed in epithelial cells of all the 10 non-malignant (histologically normal) esophageal mucosa (Figure 1A). No detectable expression of DAB2 protein was observed in 5/30 (17%) hyperplasia

Table 1 Correlation of DAB2 protein expression with clinicopathological parameters of ESCC patients

Parameters	Total cases <i>n</i>	DAB2	
		Positive <i>n</i> (%)	Negative <i>n</i> (%)
ESCC	50	16 (32)	34 (68)
Age			
≤ 40 yr	14	4 (28)	10 (72)
≥ 40 yr	36	12 (33)	24 (67)
Gender			
Male	30	11 (37)	19 (63)
Female	20	5 (25)	15 (75)
Tumor Stage			
T2	3	1	2
T3	36	11 (31)	25 (69)
T4	11	4 (36)	7 (64)
Hp Grading			
WDSCC	8	2	6
MDSCC	37	11 (30)	26 (70)
PDSCC	5	3 (60)	2 (40)
Nodal stage			
Node -ve	23	8 (35)	15 (65)
Node +ve	27	8 (30)	19 (70)
Normal	10	10	
Hyperplasia	30	25 (83)	5
Dysplasia	15	5	10 (67)

(Figure 1B), 10/15 (67%) dysplasia (Figure 1C) and 34/50 (68%) ESCCs (Figure 1D). Only 32% of ESCCs showed weak to moderate DAB2 staining (Figure 1E). Significant loss of DAB2 protein expression was observed from esophageal normal mucosa to hyperplasia, dysplasia and SCC ($P_{\text{trend}} < 0.001$). Significantly higher proportion of dysplastic tissues showed loss of DAB2 expression in comparison with hyperplasia ($P = 0.002$; OR = 9.998; 90% CI = 2.368-42.210).

DAB2 exon1 methylation in ESCCs

To determine the possibility of promoter methylation-mediated gene silencing of *DAB2*, methylation status of putative promoter in exon1 of *DAB2* gene was analyzed in 10 DAB2 immuno-negative ESCC tissues. Burkitt's lymphoma cell line Raji was used as a positive control for hypermethylation of *DAB2* gene, as reported by Akiyama *et al.*^[28]. As shown in Figure 2, 8 of 10 (80%) ESCCs showed signal (PCR amplicon) for the unmethylated *DAB2* alleles, while 2 cases showed the presence of both unmethylated and methylated PCR amplicon, suggesting that *DAB2* promoter hypermethylation may account for loss of DAB2 protein in these two tumors.

DISCUSSION

In this study, we demonstrated, probably for the first time, significant loss of DAB2 protein in different stages of development and progression of ESCC, from normal to hyperplasia, dysplasia and invasive cancer ($P_{\text{trend}} < 0.001$). Strong cytoplasmic expression of DAB2 protein was observed in all the 10 histologically non-malignant esophageal tissues. Loss of DAB2 protein was observed in

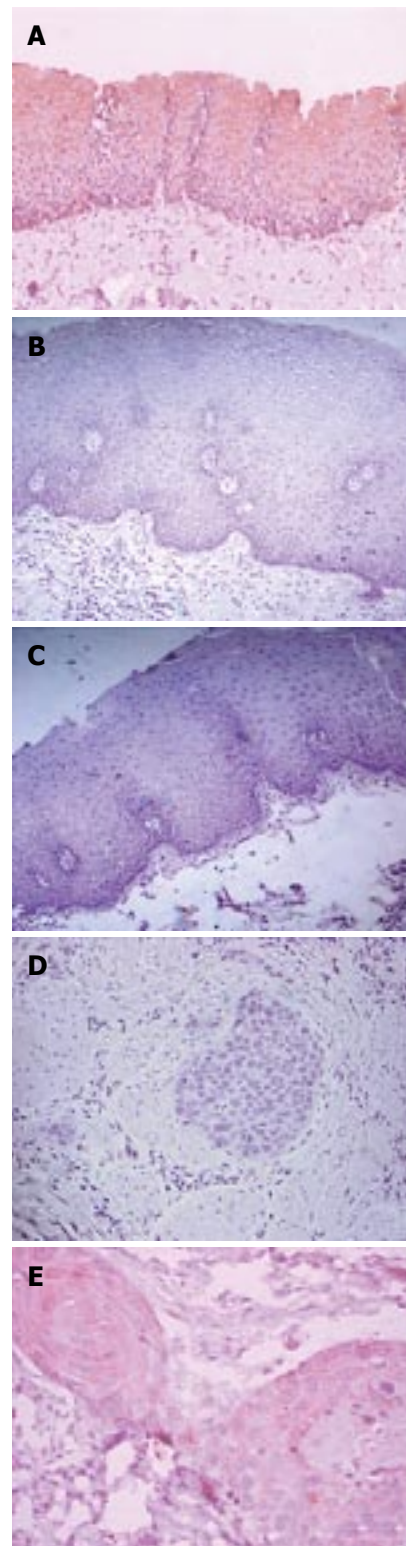


Figure 1 Immunohistochemical analysis of DAB2 in esophageal squamous cell carcinogenesis. **A:** Cytoplasmic expression of DAB2 in non-malignant normal esophageal mucosa; **B:** Hyperplastic esophageal mucosa showing no detectable expression of DAB2 protein; **C:** Dysplastic esophageal mucosa showing loss of DAB2 protein expression; **D:** ESCC section showing loss of DAB2 protein expression; and **E:** ESCC showing faint cytoplasmic DAB2 protein expression (**A-D:** Original magnification x 100; and **E:** Original magnification x 200).

preneoplastic stages, as early as in hyperplasia (in 5 of 30 hyperplastic tissues analyzed), suggesting that deregulation of DAB2 expression is likely to be an early event in esophageal tumorigenesis. Interestingly, loss of DAB2 protein was observed in significantly higher proportion of dysplastic tissues (10/15 cases) in comparison with hyperplasia ($P = 0.002$), indicating a critical impact of loss of this protein in evolution of dysplasia. Furthermore, loss of DAB2 in 34/50 (68%) of ESCCs observed in this study suggests that down-regulation of DAB2 protein is sustained down the tumorigenic pathway. To our

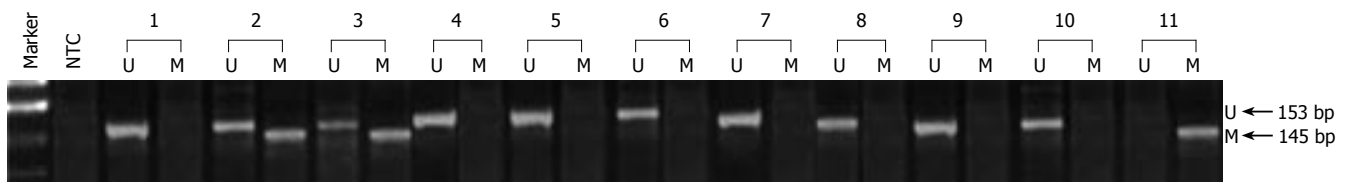


Figure 2 DAB2 exon-1 promoter hypermethylation. Methylation-specific PCR was done to determine the possibility of promoter methylation-mediated gene silencing of *DAB2*. Eight of 10 ESCCs showed signal (PCR amplicon) for the unmethylated *DAB2* alleles (samples 1, 4-10), while 2 cases showed the presence of both unmethylated and methylated PCR amplicon (samples 2 and 3). Burkitt's lymphoma cell line Raji was used as a positive control for hypermethylation of *DAB2* gene (sample 11). In each sample, lane U represents the unmethylated product, while M represents the methylated product, lane NTC refers to the no template control.

knowledge, the clinical relevance of *DAB2* in primary human esophageal tumors remains to be determined. Therefore, our study is important in demonstrating the clinical significance of aberrant *DAB2* expression in primary ESCCs.

The physical interaction of epithelial cells with the basement membrane ensures correct positioning and acts as a survival factor for epithelial cells. Cells that detach from the basement membrane often undergo apoptosis^[29,30]. In tumors, this positional control is absent, resulting in disorganized cell proliferation^[26]. Inactivation of a gene(s) controlling epithelial cell positioning may be a step in tumorigenicity. One of these genes is *DAB2*, which functions in cell positioning control and loss of *DAB2* protein has been suggested to contribute to the basement membrane-independent, disorganized proliferation of tumor cells^[26]. *DAB2* expression in breast cancer cells resulted in sensitivity to suspension-induced cell death (anoikis)^[31]. Loss of *DAB2* expression and the loss of collagen IV and laminin-containing basement membrane are two critical events associated with morphologic dysplastic changes of the ovarian surface epithelium as a step in tumorigenicity^[32,33].

Basement membrane (BM) can regulate differentiation, proliferation and polarity of esophageal epithelium and its integrity is important for carcinogenesis. Studies aimed to explore the effect of the BM changes induced by chronic inflammation on esophageal carcinogenesis have suggested that BM changes with aberrant proliferation of esophageal epithelia^[34]. The most salient finding of our study is the significant loss of *DAB2* protein expression in pre-neoplastic lesions, such as dysplasia in comparison with non-malignant esophageal epithelium and hyperplasia ($P = 0.002$). Based on the studies in ovarian cancer and transitional cell carcinoma and our observations in esophageal dysplasia and ESCC, we hypothesize that down-regulation of *DAB2* protein in dysplasia may be an important step in loss of epithelial cell positioning, aberrant proliferation and tumorigenicity. Therefore, loss of *DAB2* protein may serve as a candidate molecular marker for pre-neoplastic lesions.

Akiyama *et al*^[28] showed epigenetic silencing of *GATA-4* and *GATA-5* but not *GATA-6* transcription factor genes and their potential downstream anti-tumor target genes in colorectal and gastric cancer. *GATA6* and histone deacetylase inhibitor synergistically induce *DAB2* gene expression in transitional cell carcinoma (TCC) cell lines^[35]. Histone acetylation status associated with the 5' upstream regulatory sequence of *DAB2* gene is

one of the key determinants of its activity. *GATA6* can specifically induce *DAB2* promoter activity. Increased histone acetylation and the presence of *GATA6* have a synergistic effect on *DAB2* promoter activity which results in elevation of *DAB2* protein expression. Although the underlying mechanism leading to high *GATA6* and/or acetyl H3 levels in TCC cell lines is still unclear, it is likely that enzymes responsible for epigenetic regulation, such as histone modification or DNA methylation, could play a role. Therefore, we analyzed the methylation status of *DAB2* gene in esophageal tumors that showed loss of *DAB2* protein. Methylation-specific PCR showed methylation of *DAB2* promoter in 2 of the 10 *DAB2* immuno-negative ESCCs analyzed. These findings suggest that epigenetic silencing of *DAB2* is infrequent in ESCCs and accounts for down-regulation of the protein in only a subset of esophageal tumors. Thus, there is a need to investigate other mechanisms that may be responsible for loss of *DAB2* protein in ESCCs harboring the unmethylated *DAB2* promoters. A parallel study on *DAB2* protein expression and promoter methylation in our laboratory showed similar discordance between loss of protein expression and epigenetic silencing of the gene in breast cancer. *In silico* analysis suggested that post-transcriptional, micro RNA-mediated targeting of *DAB2* mRNA may be another mechanism for gene silencing (unpublished data of Bagadi SAR *et al*). It will be worthwhile to determine if micro RNA is involved in targeting of *DAB2* mRNA accounting for loss of *DAB2* protein in ESCCs as well.

In conclusion, our data suggest that loss of *DAB2* protein occurs in early pre-neoplastic stages and is sustained down the tumorigenic pathway of esophageal squamous cell carcinogenesis, underscoring its potential as a candidate molecular marker for pre-neoplastic lesions. Furthermore, *DAB2* exon-1 promoter hypermethylation is an infrequent event in ESCCs.

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RAPID COMMUNICATION

Inhibition of hepatitis B virus expression and replication by RNA interference in HepG2.2.15

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Abstract

AIM: To observe the inhibition of hepatitis B virus replication and expression by transfecting vector-based small interference RNA (siRNA) pGenesil-HBV X targeting HBV X gene region into HepG2.2.15 cells.

METHODS: pGenesil-HBV X was constructed and transfected into HepG2.2.15 cells *via* lipofection. HBV antigen secretion was determined 24, 48, and 72 h after transfection by time-resolved immunofluorometric assays (TRFIA). HBV replication was examined by fluorescence quantitative PCR, and the expression of cytoplasmic viral proteins was determined by immunohistochemistry.

RESULTS: The secretion of HBsAg and HBeAg into the supernatant was found to be inhibited by 28.5% and 32.2% ($P < 0.01$), and by 38.67% ($P < 0.05$) and 42.86% ($P < 0.01$) at 48 h and 72 h after pGenesil-HBV X transfection, respectively. Immunohistochemical staining for cytoplasmic HBsAg showed a similar decline in HepG2.2.15 cells 48 h after transfection. The number of HBV genomes within culture supernatants was also significantly decreased 48 h and 72 h post-transfection as quantified by fluorescence PCR ($P < 0.05$).

CONCLUSION: In HepG2.2.15 cells, HBV replication and expression is inhibited by vector-based siRNA pGenesil-HBV X targeting the HBV X coding region.

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Key words: Hepatitis B virus; RNA interference; Plasmid vector; HepG2.2.15

Zhao ZF, Yang H, Han DW, Zhao LF, Zhang GY, Zhang Y, Liu

INTRODUCTION

Hepatitis B is a severe infectious disease threatening peoples' health all over the world. There is still no efficient therapy to control HBV persistent replication, which may lead to the development of liver cirrhosis and hepatocellular carcinoma (HCC)^[1]. RNA interference (RNAi) is a highly specific and effective mechanism of post-transcriptional gene silencing mediated by double-stranded RNA of 21-23 nt in size. Several researches have suggested that RNAi could provide a new therapeutic strategy against chronic HBV infection^[2-6]. In the present study, a plasmid leading to the expression of small interfering RNA (siRNA) that targets the HBV X gene was transfected into HepG2.2.15 cells, and HBV DNA replication as well as HBV antigen expression and secretion were monitored.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL, USA. Metafectene transfection reagent was purchased from Germany Biontes. Diagnostic kits for HBsAg and HBeAg (time-resolved immunofluorometric assay) were obtained from Suzhou Xinbo Biotechnology Corporation. Mouse monoclonal antibody directed against human HBsAg and rabbit anti-mouse horseradish peroxidase (HRP)-IgG were purchased from Beijing Zhongshan Golden Bridge Biotechnology Corporation. HepG2.2.15 cells were obtained from the Institute of Infectious Disease, Beijing University Medicine School.

Plasmid construction

pGenesil, containing human U6 promoter, was used to generate a series of siRNA expression vectors by inserting annealed oligonucleotides between *Bam*HI and *Hind*III sites. The oligonucleotides 5'-GAT CCG GTC TTA CAT AAG AGG ACT TTC AAG ACG AGT CCT CTT ATG TAA GAC CTT TTT TGT CGA CA-3' (sense) and 3'

Table 1 Effect of pGenesil-siHBV X on HBsAg and HBeAg expression transfected HepG2.2.15 cells

Group	HBsAg ($\mu\text{g/L}$)			HBeAg (Ncu/mL)		
	24 h	48 h	72 h	24 h	48 h	72 h
Untreated	7.13 \pm 0.20	14.43 \pm 0.56	22.50 \pm 2.14	0.43 \pm 0.02	0.75 \pm 0.06	1.12 \pm 0.10
Metafectene	6.80 \pm 0.17	13.57 \pm 0.88	21.84 \pm 0.91	0.48 \pm 0.02	0.69 \pm 0.08	1.03 \pm 0.06
pGenesil	6.57 \pm 0.40	14.30 \pm 0.47	24.15 \pm 2.24	0.45 \pm 0.06	0.64 \pm 0.05	1.00 \pm 0.06
PGenesil-HK	6.47 \pm 0.20	13.63 \pm 0.64	21.84 \pm 0.51	0.46 \pm 0.05	0.69 \pm 0.07	0.97 \pm 0.08
PGenesil-AFP	6.33 \pm 0.35	13.70 \pm 0.73	23.11 \pm 1.25	0.47 \pm 0.05	0.76 \pm 0.08	1.10 \pm 0.12
PGenesil-HBVX	5.97 \pm 0.13	10.25 \pm 0.32 ^b	15.26 \pm 0.88 ^b	0.43 \pm 0.02	0.46 \pm 0.01 ^c	0.64 \pm 0.04 ^c

^b $P < 0.01$ vs untreated control and other controls; ^c $P < 0.05$ vs untreated control and other controls.

-GCC AGA ATG TAT TCT CCT GAA AGT TCT GCT CAG GAG AAT ACA TTC TGG AAA AAA CAG CTG TTC GA-5' (antisense) were used for the construction of pGenesil-HBV X targeting HBV X (N 1649 to 1667)^[7]; 5'-GAT CCG CAT TG G CAA AGC GAA GCT TTC AAG ACG AGC TTC GCT TTG CCA ATG CTT TTT TGT CGA CA-3' (sense) and 3'-GCG TAA CCG TTT CGC TTC GAA AGT TCT GCT CGA AGC GAA ACG GTT ACG AAA AAA CAG CTG TTC GA-5' (antisense) for a control vector targeting the α -fetoprotein (AFP) gene (N 1275 to 1293); and 5'-GAT CCG ACT TCA TAA GGC GCA TGC TTC AAG ACG GCA TGC GCC TTA TGA AGT CTT TTT TGT CGA CA-3' (sense) and 3'-GCT GAA GTA TTC CGC GTA CGA AGT TCT GCC GTA CGC GGA ATA CTT CAG AAA AAA CAG CTG TTC GA-5' (antisense) for the control vector pGenesil-HK producing a random sequence of siRNA.

Cell culture and transfection

HepG2.2.15 cells were maintained in DMEM supplemented with 100 mL/L fetal calf serum (FCS), 100 IU/mL penicillin, 100 mg/L streptomycin and 2 mmol/L L-glutamine at 37°C in an atmosphere of 50 mL/L CO₂. The cells were plated in 6-well plates which had been placed sterile cover slips (1×10^6 cells per well). Transfection was performed at about 70% confluence with pGenesil and Metafectene lipofection reagent complex at a ratio of 8 μL :2.5 μg . Under these conditions we obtained a 55% transfection efficiency (data not shown).

Quantitative assay of HBsAg and HBeAg

The levels of HBsAg and HBeAg in culture supernatants were measured at 24, 48 and 72 h after transfection by using time-resolved immunofluorometric assay kits (TRFIA) according to the supplier's instructions.

Assay of HBV DNA replication

HepG2.2.15 cells were harvested at 24, 48, and 72 h post-transfection. Forty μL of the supernatant were mixed with an equal volume of the DNA extractant. Samples were boiled for 10 min and then centrifuged at $10000 \times g$ for 5 min. Two μL of the samples were transferred into PCR reaction tubes. PCR cycling parameters consisted of denaturation at 93°C for 2 min, followed by 93°C for 45 s; 55°C for 60 s \times 10 cycles and then 93°C for 30 s; 55°C for 45 s \times 30 cycles.

Immunocytochemistry of HBsAg

To examine whether the effects of pGenesil-HBV X on HBsAg production were uniform with the culture media, cytoplasmic HBsAg was visualized by indirect immunocytochemistry 48 h post-transfection. pGenesil-HBV X transfected and control cells were washed with PBS, fixed in 900 mL/L ethanol for 10 min at room temperature and then washed with PBS. The fixed cells were permeabilized with 5 mL/L Triton X-100 in PBS for 15 min at 37°C and washed with PBS. To inhibit endogenous peroxidase, cells were exposed to 3 mL/L hydrogen peroxide for 10 min at 25°C. After washed with PBS, cells were incubated with mouse monoclonal anti-HBsAg antibody for 2 h at 37°C and subsequently with rabbit anti-mouse IgG conjugated horseradish-peroxidase for 30 min at 37°C. Cells were visualized with 3, 3'-diaminobenzidine tetrahydrochloride substrate and examined by light microscopy.

Statistical analysis

All statistical analysis were performed using the Microsoft SPSS 12.0 software. The graphs represented in mean \pm SD and compared using unpaired t-test. $P < 0.05$ was regarded as a significant difference.

RESULTS

pGenesil-HBV X inhibited HBsAg and HBeAg secretion in cultured HepG2.2.15

HBsAg and HBeAg concentrations were measured in cell culture supernatants of pGenesil-HBV X treated and control cells 24, 48, and 72 h post-transfection by using TRFIA (Table 1). At 24 h in the culture media, there was no significant difference between pGenesil-HBV X treated cells and other controls (untreated control, pGenesil-AFP control, pGenesil-HK control, pGenesil alone and Metafectene alone) ($P > 0.05$), while HBsAg was inhibited at 48 and 72 h by 28.47% and 32.16% ($P < 0.01$). HBeAg was reduced at 48 and 72 h post-transfection ($P < 0.05$) by 38.7% and 42.9% in the media of pGenesil-HBV X treated cells compared to the controls.

Inhibited HBV DNA replication in cultured HepG2.2.15

Levels of HBV DNA were examined by fluorescence quantitative PCR. This assay can detect HBV DNA in the range of 10^3 to 10^8 copies. The results revealed a

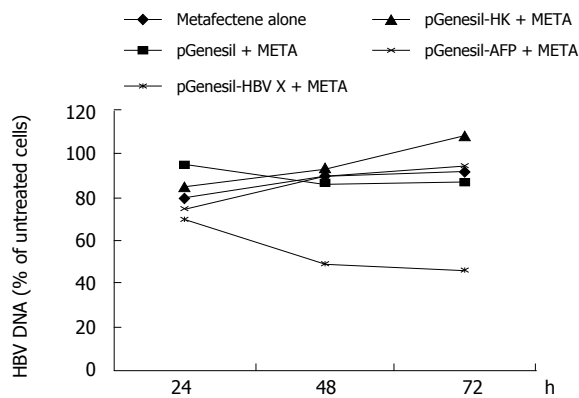


Figure 1 Inhibition of pGenesil-HBV X on HBV DNA in HepG2.2.15.

significant decrease in DNA replication when pGenesil-HBV X treated cells were compared to untreated cells. The number of HBV DNA copies in pGenesil-HBV X treated cells was found to be reduced by 44.9% and 45.9% at 48 and 72 h after transfection, respectively ($P < 0.05$), while the other controls showed no significant difference to the untreated cultures at any time point (Figure 1).

The effects of pGenesil-HBV X on intracellular HBsAg in HepG2.2.15

The effects of pGenesil-HBV X on intracellular HBsAg were visualized by immunocytochemistry 48 h after transfection. Intracellular HBsAg is localized in the cytoplasm of HepG2.2.15 cells at normal. In cells treated with pGenesil-HBV X, HBsAg was either decreased or non-detectable. In contrast, cells of other controls were obviously stained (Figure 2).

DISCUSSION

RNAi is a mechanism of post-transcriptional gene silencing mediated by double-stranded RNA of 21-23 nt in length. RNAi has been applied as a highly specific and efficient tool to interfere with viral replication as it has been shown for HIV^[8-12], hepatitis C virus^[13-16], myxovirus^[17], gamma herpesvirus^[18] or influenza virus^[19]. HBV DNA replication requires reverse transcription to form a pregenomic RNA that is similar to reverse-transcription virus. 3.2kb pregenomic mRNA is not only translated into HBV proteins including HBeAg, HBcAg and HBV DNAP, but it is also a template for the synthesis of viral DNA to continue replication. Furthermore, all four transcripts include the HBV X protein coding region. Selecting a conserved sequence in the X gene region as a target, we expect to inhibit the expression of HBV antigens and the replication of HBV DNA. To set up an in vitro model which is more stable and more similar to natural condition of viral infection is a base of experiment and is a key prerequisite to evaluate the efficiency of anti-virus therapy. Previous studies were carried out by co-transfecting siRNA or siRNA expression vector and a plasmid cloned with full-length HBV DNA or HBV target region into cells^[7,20,21]. It is obvious that cells transfected with siRNA or siRNA expression vectors must get HBV

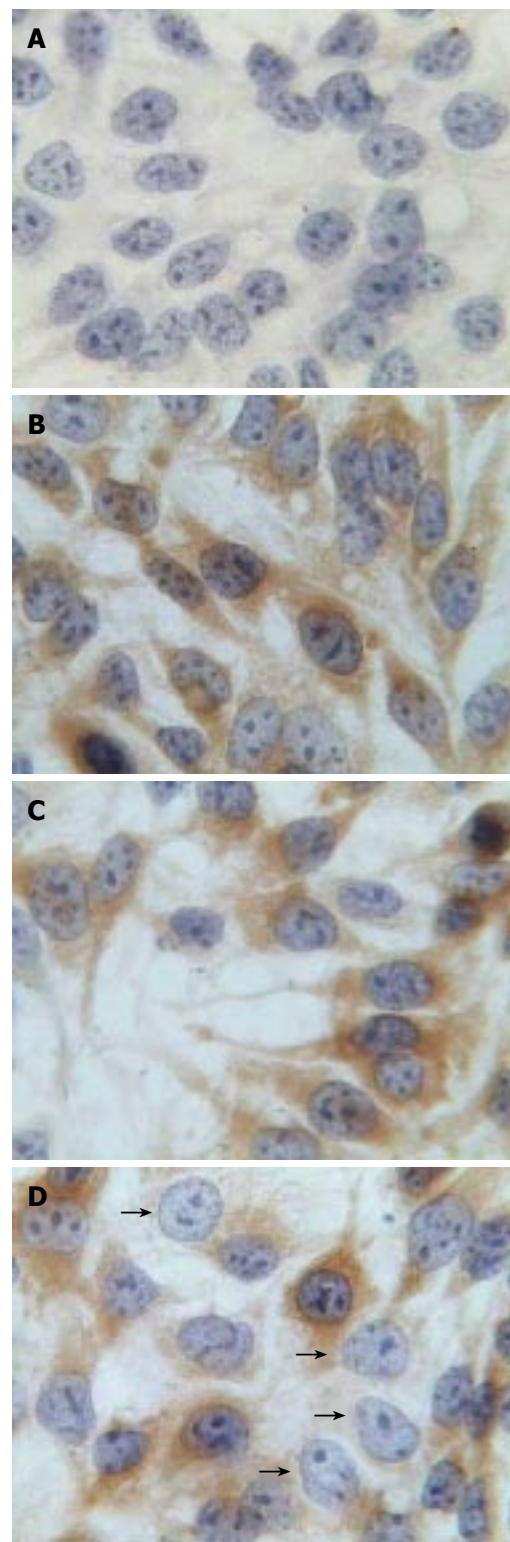


Figure 2 Immunocytochemistry for intracellular HBsAg in HepG2.2.15 ($\times 400$). A: Without monoclonal antibody against HBsAg; B: pGenesil-HK treated cells; C: pGenesil-AFP treated cells; D: pGenesil-HBV X treated cells. Arrows show the cells were transfected and HBsAg expression was suppressed.

expression plasmid at same time, which is better to observe and evaluate the specificity and efficiency by siRNA. But only selecting out the cells co-transfected successfully for research does not coincide with natural conditions of viral infection and will not reflect the exact effects of siRNA on HBV target gene.

The HepG2.2.15 cell line, a derivative of the human HepG2 hepatoma cell line that has been stably transformed with a head-to-tail dimer of HBV DNA^[22], was chosen as a model because it produces HBV infectious particles constitutively and expresses HBV antigens stably. To transfected siRNA expression vector into HepG2.2.15 cells, and culturing transfected cells and untransfected cells under one system, we could simulate the nature condition that virus still replicate and express constitutively in untransfected cells. As a result, we found that HBsAg and HBeAg in the supernatant were inhibited by 28.5% and 38.7% at 48 h, and decreased by 32.2% and 42.9% 72 h post-transfection with pGenesil-HBV X against HBV X. Levels of HBV DNA were also found to be reduced. Moreover, immunocytochemistry revealed that the amount of intracellular HBsAg parallels the decline in HBV serum markers in cultures treated with pGenesil-HBV X. Controls (including untreated cells and treated with Metafectent reagent alone, pGenesil plasmid alone, pGenesil-AFP or pGenesil-HK expressing random siRNA) failed to reduce HBV expression and replication. At the premises of targeting same sequence in HBV X region, the efficiency we got was lower than that of Shlomai^[7] who used a co-transfection approach to Huh-7 cells. At present, there isn't any reagent that achieves 100% transfection efficiency neither to cells nor to animals. Selecting HepG2.2.15 cell line as a model is better for us to evaluate the effects of siRNA in consideration of the efficiency of transfection, and thus it will be valuable for us to evaluate the effects of siRNA on clinical application research in the future.

By measuring the levels of AFP which is constitutively secreted by HepG2.2.15 cells no change was found between pGenesil-HBV X treated cells and other controls except pGenesil-AFP treated cells. This result shows that pGenesil-HBV X specifically inhibits HBV gene expression.

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RAPID COMMUNICATION

Expression of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 in ulcerative colitis

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Abstract

AIM: To examine the expression of metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in the colonic mucosa of patients with ulcerative colitis (UC).

METHODS: Reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry were used to study the expression of MMP-1 and TIMP-1 at both mRNA and protein levels in patients with UC and controls. The relationship between MMP-1 mRNA, TIMP-1 mRNA, MMP-1 mRNA/TIMP-1 mRNA ratio and the severity of clinical symptoms of the patients with UC were also analyzed.

RESULTS: The expression of MMP-1 mRNA and TIMP-1 mRNA in the ulcerated and inflamed colonic mucosa was significantly higher than that in the non-inflamed colonic mucosa ($P < 0.001$), but there was no statistically significant difference in the non-inflamed colonic mucosa of UC patients and normal controls ($P > 0.05$). The mRNA expression of MMP-1 and TIMP-1 in ulcerated colonic mucosa of UC patients was increased by 80-fold and 2.2-fold, respectively when compared with the normal controls. In the inflamed colonic mucosa, the increase was 30-fold and 1.6-fold, respectively. Immunohistochemical analysis showed that among the ulcerated, inflamed, and non-inflamed colonic mucosae of UC patients and the normal controls, the positive rate of MMP-1 expression was 87%, 87%, 40% and 35% respectively, and the positive rate of TIMP-1 expression was 89%, 89%, 80% and 75%, respectively. Furthermore, the expression of MMP-1 mRNA, TIMP-1 mRNA and the MMP-1 mRNA/TIMP-1 mRNA ratio were correlated with the severity of clinical symptoms ($P < 0.05$).

CONCLUSION: Excessive expression of MMP-1 in the diseased colonic mucosa causes excessive hydrolysis of the extracellular matrix (ECM) and ulceration in UC pa-

tients. MMP-1 mRNA, TIMP-1 mRNA and MMP-1 mRNA/TIMP-1 mRNA ratio can be used as biomarkers to judge the severity of clinical symptoms in patients with UC. Exogenous TIMP-1 or MMP-1 inhibitor therapy is a novel treatment for patients with UC.

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Key words: Matrix metalloproteinase-1; Tissue inhibitor of metalloproteinase-1; Ulcerative colitis; Reverse transcription-polymerase chain reaction; Immunohistochemistry

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<http://www.wjgnet.com/1007-9327/12/6050.asp>

INTRODUCTION

Ulcerative colitis (UC) is a chronic, non-specific inflammatory disease of the colonic mucosa with unknown etiology and pathogenesis. Pathologically, it is characterized by ulceration in the mucosal and submucosal areas, and degradation of extracellular matrix (ECM) is one of the major events during this process^[1]. Matrix metalloproteinase-1 (MMP-1) produced by cytokine-activated interstitial cells is one of the most important enzymes in degrading ECM, and the activity of MMP-1 is controlled by its natural inhibitor, tissue inhibitor of metalloproteinase (TIMP-1)^[2]. Therefore, in this study we measured MMP-1 and TIMP-1 transcripts and their proteins by using reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry to explore their possible role in patients with UC.

MATERIALS AND METHODS

Patients and samples

Thirty patients with UC confirmed by colonoscopy and biopsy were enrolled in the study. Among these patients, 21 were males and 9 were females with their age ranged from 18 to 76 years and averaged 48 years. Samples were taken from the ulcerated, inflamed and non-inflamed areas of the colonic mucosa during colonoscopy. There were 3 patients with pan-colonic lesions, 2 with hemi-colonic lesions, 13 with recto-sigmoidal lesions, and 12 with rectal lesions. Based on the clinical manifestations, 2 patients

were classified into severe type, 18 into moderate type, and 10 into mild type. Meanwhile, 20 normal subjects were selected as controls; 14 of them were males and 6 were females with their age ranged from 27 to 65 years and averaged 46 years. Biopsy samples were immediately snap frozen in liquid nitrogen and stored at -80°C for RT-PCR. Separate biopsy samples were fixed in formalin and embedded in paraffin for immunohistochemistry.

Total RNA extraction

Total RNA was extracted from the frozen samples using the RNA isolation kit (Invitrogen Company) following the manufacturer's instructions. Five μ L of the extracted RNA was run on 1% agarose gel electrophoresis to identify the extracted products.

RT-PCR for MMP-1 and TIMP-1

RT-PCR was performed using the TaKaRa RNA PCR kit 3.0 (AMV) (supplied by Dalian Baosheng Biotechnology Company) following the manufacturer's instructions. Primer sequences^[3] used were as follows: MMP-1: sense: 5'-ATGCGAACAATCCCTTCTACC-3', antisense: 5'-T'TCCCTCAGAAAGAGCAGCATCG-3'; TIMP-1: sense: 5'-GGACACCAGAAGTCAACCAGCC-3', antisense: 5'-CGTCCACAAGCAATGAGTCC-3'. Primers for β -actin were used as the internal control: sense: 5'-CCTTCCTGGCATGGAGTCCTG-3', antisense: 5'-GGAGCAATGATCTTGATCTTC-3'. Reverse transcription was carried out at 30°C for 10 min, at 42°C for 30 min, at 99°C for 5 min, and at 5°C for 5 min. PCR was performed as follows: initial denaturation at 94°C for 2 min, 35 amplification cycles at 94°C for 30 s, at 53°C for 30 s, at 72°C for 1 min, extension at 72°C for 10 min. Five μ L PCR product was run on 2% agarose gel electrophoresis.

Immunohistochemistry

Sample sections were washed 3 times with PBS, 3 min each time after initial treatment. Primary antibodies, rabbit anti-human MMP-1 polyclonal antibody and TIMP-1 monoclonal antibody (Beijing Zhongshan Biology Company) were added and incubated at room temperature for 1.5 h, washed again and incubated with peroxidase-conjugated secondary antibody for 15 min and washed again. A brown product was developed in diaminobenzidine (DAB) for 10 min.

Result determination and statistical analysis

Bio-imaging system (PALI Company, USA) was employed to analyze the density of the bands of PCR products. MMP-1 mRNA and TIMP-1 mRNA were semi-quantitatively expressed by the ratios between MMP-1, TIMP-1 and β -actin OD values. All values were expressed as mean \pm SD.

Results of immunohistochemistry were scored according to the degree of staining and percentage of positive cells as no staining: 0 point, mild staining: 1 point, moderate staining: 2 points, heavy staining: 3 points; positive cells \leq 5%: 0 point, 6%-30%: 1 point, 31%-70%: 2 points, 71%-100%: 3 points. Final score was determined by combining points obtained from the above two scoring

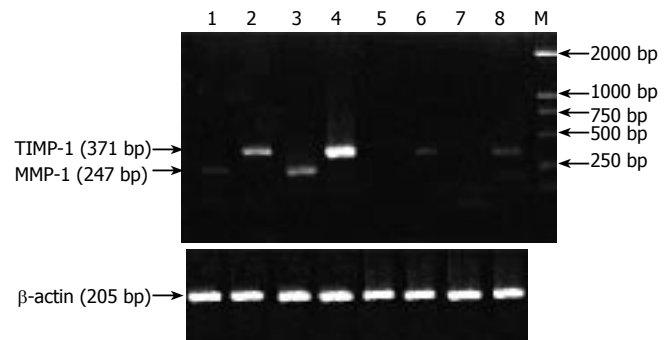


Figure 1 Expression of MMP-1 and TIMP-1 mRNA in UC. Lanes 1, 2: Inflamed area; lanes 3, 4: Ulcerated area; lanes 5, 6: Non-inflamed area; lanes 7, 8: Normal controls; M: DNA marker DL 2000.

systems: 0-1 point: (-), 2 points: (+), 3-4 points: (++), 5-6 points: (+++).

Student-Neuman-Keuls test was used to compare MMP-1 mRNA and TIMP-1 mRNA in different colon biopsy samples. Positive rates were analyzed by χ^2 test and Spearman correlation analysis was used to study the relationship between MMP-1 mRNA, TIMP-1 mRNA, MMP-1 mRNA/TIMP-1 mRNA ratio and the severity of clinical symptoms. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 10.0 for Windows.

RESULTS

Expression of MMP-1 mRNA in UC

Expression of MMP-1 mRNA in the ulcerated and inflamed areas of the colon was significantly higher than that in the non-inflamed areas of the colon of UC patients and normal controls ($P < 0.001$). It was 80-fold higher in the ulcerated area and 30-fold higher in the inflamed area when compared with the normal controls, but there was no statistically significant difference in MMP-1 mRNA expression between UC patients and normal controls ($P > 0.05$, Figure 1, Table 1).

Expression of TIMP-1 mRNA in UC

Expression of TIMP-1 mRNA in the ulcerated and inflamed area of colon was significantly higher than that in the non-inflamed area of colon of UC patients and normal controls ($P < 0.001$). It was 2.2-fold higher in the ulcerated area and 1.6-fold higher in the inflamed area when compared with the normal controls, but there was no statistically significant difference in TIMP-1 mRNA expression between UC patients and normal controls (Figure 1, Table 2).

Correlations between MMP-1mRNA, TIMP-1mRNA, MMP-1 mRNA/TIMP-1 mRNA ratio and the severity of clinical symptoms

Expression of MMP-1 mRNA was significantly related to the expression of TIMP-1 mRNA in the tissues of UC patients, the relating coefficient was 0.801 ($P < 0.001$). Based on clinical manifestations, only 2 patients were classified into severe type, so correlation analysis was performed between moderate group (18 patients) and mild group

Table 1 Expression of MMP-1 mRNA in samples from different areas of colon in UC (mean \pm SD)

Samples	MMP-1 mRNA	P value
Ulcerated area	0.4136 \pm 0.2495	< 0.001 ^{b,d}
Inflamed area	0.1491 \pm 0.0891	< 0.001 ^{b,d} < 0.05 ^a
Non-inflamed area	0.0102 \pm 0.0144	> 0.05
Normal controls	0.0051 \pm 0.0086	

^b*P* < 0.001 *vs* normal controls, ^d*P* < 0.001 *vs* non-inflamed area, ^a*P* < 0.05 *vs* ulcerated area.

Table 3 Positive expression rates of MMP-1 in samples from different areas of colon in UC

Samples	Positive cases	Negative cases	Total cases	Positive rate (%)	P Value
Ulcerated area	25	5	30	87	< 0.05 ^{a,c}
Inflamed area	25	5	30	87	< 0.05 ^{a,c}
Non-inflamed area	12	18	30	40	> 0.05
Normal controls	7	13	20	35	

^a*P* < 0.05 *vs* normal controls, ^c*P* < 0.05 *vs* non-inflamed area.

Table 5 Positive expression rates of TIMP-1 in samples of different areas of colon in UC

Samples	Positive cases	Negative cases	Total cases	Positive rate (%)	P value
Ulcerated area	26	4	30	89	> 0.05
Inflamed area	26	4	30	89	> 0.05
Non-inflamed area	24	6	30	80	> 0.05
Normal controls	15	5	20	75	

(10 patients). The results showed that MMP-1 mRNA, TIMP-1 mRNA and MMP-1 mRNA/TIMP-1 mRNA ratio were all significantly correlated with the severity of the clinical symptoms, and the relating coefficient was 0.411, 0.328 and 0.552, respectively.

Results of immunohistochemistry

Positive expression rates of MMP-1 in the ulcerated and inflamed areas of the colon were significantly higher than those in the non-inflamed areas of the colon of UC patients and normal controls (*P* < 0.05), but there was no statistically significant difference in non-inflamed areas between UC patients and normal controls (Table 3).

The intensity of MMP-1 expression in the ulcerated and inflamed area of colon was significantly higher than that in the non-inflamed area of colon of UC patients and normal controls (*P* < 0.001), but there was no statistically significant difference in the non-inflamed area between UC patients and normal controls (Table 4).

Positive expression rates of TIMP-1 in the ulcerated and inflamed area of colon were not statistically different when compared with those in the non-inflamed area of colon of UC patients and normal controls (Table 5).

The intensity of TIMP-1 expression in the ulcerated and inflamed area of colon was significantly higher than

Table 2 Expression of TIMP-1 mRNA in samples from different areas of colon in UC (mean \pm SD)

Samples	TIMP-1	P value
Ulcerated area	0.8512 \pm 0.4169	< 0.001 ^{b,d}
Inflamed area	0.7493 \pm 0.3505	< 0.05 ^{a,c,e}
Non-inflamed area	0.4434 \pm 0.3360	> 0.05
Normal control	0.3903 \pm 0.2971	

^b*P* < 0.001 *vs* normal controls, ^d*P* < 0.001 *vs* non-inflamed area, ^a*P* < 0.05 *vs* normal controls, ^c*P* < 0.05 *vs* non-inflamed area, ^e*P* < 0.05 *vs* ulcerated area.

Table 4 Intensity of MMP-1 expression in samples from different areas of colon in UC

Samples	-	+	++	+++	P value
Ulcerated area	5	2	4	19	< 0.001 ^{a,c}
Inflamed area	5	5	16	4	< 0.05 ^{a,c,e}
Non-inflamed area	18	10	2	0	> 0.05
Normal controls	13	6	1	0	

^a*P* < 0.05 *vs* normal controls, ^c*P* < 0.05 *vs* non-inflamed area, ^e*P* < 0.05 *vs* ulcerated area.

Table 6 Intensity of TIMP-1 expression in samples from different areas of colon in UC

Samples	-	+	++	+++	P value
Ulcerated area	4	7	9	10	< 0.05 ^{a,c}
Inflamed area	4	8	13	5	< 0.05 ^{a,c,e}
Non-inflamed area	6	19	5	0	> 0.05
Normal controls	5	11	4	0	

^a*P* < 0.05 *vs* normal controls, ^c*P* < 0.05 *vs* non-inflamed area, ^e*P* < 0.05 *vs* ulcerated area.

that in the non-inflamed area of colon of UC patients and normal controls (*P* < 0.001). The results also showed that the intensity of TIMP-1 expression in the ulcerated area was significantly greater than that in the inflamed area (*P* < 0.05), but there was no statistically significant difference in the non-inflamed area between UC patients and normal controls (Table 6).

DISCUSSION

UC is a chronic and non-specific inflammatory disease of the colon and affects mainly the colonic mucosa and submucosa. Pathologically, it is characterized by ulceration in the mucosa and submucosa and degradation of ECM is involved in this process. In this study, we separately utilized RT-PCR and immunohistochemistry to detect the expression of MMP-1 and TIMP-1 at both mRNA and protein levels. The expression of MMP-1 mRNA and TIMP-1 mRNA in ulcerated and inflamed areas of colon was significantly higher than that in the non-inflamed area of colon of UC patients and normal controls, the same results were also obtained at the protein level, suggesting that expression of MMP-1 and TIMP-1 is correlated to UC severity at both transcription and protein levels^[4]. MMP-1, also termed interstitial collagenase, is able to degrade the

spiral structure of collagen types I, II, III and X, making them more sensitive to the hydrolysis of gelatinase, and thus, play an important role in the degradation of ECM^[5]. RT-PCR showed that the expression of MMP-1 mRNA was greatly increased in the ulcerated and inflamed areas of the colon of UC patients with the ulcerated area being more profound, suggesting that MMP-1 is related to the mucosal damage^[6]. Arihiro *et al*^[7] showed that MMP-1 has something to do with the initial steps of ulceration in UC. von Lampe *et al*^[3] also found that the expression of MMP-1 is increased by 230-fold in the colonic mucosa of patients with UC compared with normal controls. Meanwhile, our immunohistochemical results showed that MMP-1 protein was significantly increased in the ulcerated and inflamed areas of UC patients. Furthermore, MMP-1 protein was mainly expressed in the interstitial cells. This is similar to the result of von Lampe *et al*^[3] who identified that these interstitial cells are macrophages. Mckaig *et al*^[8] identified that myofibroblasts are also MMP-1 producing interstitial cells in the colon of patients with UC.

TIMPs are natural inhibitors of MMPs and exert important regulating functions on MMPs. TIMP-1 mainly inhibits the activity of MMP-1, -3 and -9. Our results showed that the expression of TIMP-1 mRNA in the ulcerated and inflamed areas was significantly higher than that in the normal controls, suggesting that the increased expression of MMP-1 up-regulates TIMP-1, leading to an imbalanced state of MMP-1 mRNA/TIMP-1 mRNA ratio, or in other terms, the increased TIMP-1 mRNA was not able to counteract the increased MMP-1 mRNA, resulting in over degradation of ECM in UC. The similar result was also obtained when TIMP-1 protein was measured. Both MMP-1 and TIMP-1 were expressed in the interstitial cells, indicating that MMP-1 and TIMP-1 come from the same cells^[2]. Immuno-electron microscopy reveals that MMP-1 and TIMP-1 are localized in the rough endoplasmic reticulum of the activated myofibroblasts and smooth muscle cells of the blood vessels^[7], suggesting that MMP-1 and TIMP-1 have something to do with the formation of new blood vessels.

Based on clinical manifestations, only 2 UC patients were classified as severe type in our study. We analyzed the relationship between MMP-1 mRNA and TIMP-1 mRNA expression and the severity of the disease. Our results showed that expression of MMP-1 mRNA and TIMP-1 mRNA in patients with moderate UC was significantly higher than that in patients with mild UC. MMP-1 mRNA, TIMP-1 mRNA and MMP-1 mRNA/TIMP-1 mRNA ratio all were closely related to the severity of the clinical

manifestations. Therefore, it is concluded preliminarily that MMP-1 mRNA, TIMP-1 mRNA and MMP-1 mRNA/TIMP-1 mRNA ratio can be used as the parameters in judging the clinical severity of patients with UC. von Lampe *et al*^[3] found that MMP-1 mRNA expression is correlated with the pathological staging of inflammation. Wiercinska-Drapalo *et al*^[9] found that serum TIMP-1 level is positively correlated with the extent of endoscopic mucosal injury, clinical severity and concentration of C reactive protein in UC patients.

In conclusion, excessive expression of MMP-1 in the diseased colon mucosa of UC patients causes excessive hydrolysis of the ECM and ulceration. MMP-1 mRNA, TIMP-1 mRNA and MMP-1 mRNA/TIMP-1 mRNA ratio can be used as biomarkers to judge the severity of clinical symptoms in patients with UC. Exogenous TIMP-1 or MMP-1 inhibitor therapy is a novel treatment for patients with UC.

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RAPID COMMUNICATION

Genotype-dependent activation or repression of HBV enhancer II by transcription factor COUP-TF1

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Abstract

AIM: To study the expression of HBV enhancer II by transcription factor COUP-TF1.

METHODS: In order to study the regulation of HBV variants in the vicinity of the NRRE we cloned luciferase constructs containing the HBV enhancer II from variants and from HBV genotypes A and D and cotransfected them together with expression vectors for COUP-TF1 into HepG2 cells.

RESULTS: Our findings show that enhancer II of HBV genotype A is also repressed by COUP-TF1. In contrast, two different enhancer II constructs of HBV genotype D were activated by COUP-TF1. The activation was independent of the NRRE because a natural variant with a deletion of nt 1763-1770 was still activated by COUP-TF1.

CONCLUSION: Regulation of transcription of the HBV genome seems to differ among HBV genomes derived from different genotypes. These differences in transcriptional control among HBV genotypes may be the molecular basis for differences in the clinical course among HBV genotypes.

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Key words: Hepatitis B virus; Hepatitis B virus x protein;

INTRODUCTION

Hepatitis B virus (HBV) is a major health burden for the world^[1]. In an otherwise immune competent host, HBV concentrations of 10^{12} to 10^{13} genome equivalents (GE)/l are frequently found. Thus, in a chronic carrier up to 10^{13} virions are produced per day^[2]. Due to the high replication capacity^[2,3] and the high error rate of the viral polymerase, HBV genomes with all possible single mutations and double mutations of every nucleotide of the HBV genome are produced every day^[3]. Variants of HBV with point mutations, deletions or insertions have been described all over the genome of HBV^[4-7]. In addition to viral variants the variability of the virus is increased by the divergence of HBV into 8 genotypes A-H that differ by at least 8% when comparing whole genomes^[8-10]. The transcription of the hepadnaviral pregenomic (pG) and precore (pC) RNA is regulated by transcription factors binding to a region containing the enhancer II (EII) and the core promoter (Figure 1A)^[11]. The expression of both pG and pC RNA is tightly coupled^[11], however, natural variants^[12] and point mutations^[13] located in this transcriptional element can uncouple the transcription of these RNAs. The variants and artificial point mutations that uncouple the transcription of the pG and pC RNA affect a site in this transcriptional element called the nuclear receptor responsive element (NRRE)^[13,14]. COUP-TF, a member of the nuclear receptor family^[15], binds to the NRRE and has been found to repress transcription from HBV enhancer II/pC/pG promoter^[13].

We have previously characterised an outbreak of HBV in children in a department of oncology^[16,17]. In 1992 we observed a deletion of nt 1763 to 1770 affecting the c-terminus of HBx, enhancer II/pC- and pG promoter^[18] in serum from a single patient. Later on when we examined serum from 20 patients by amplification of the whole genome from different time points after infection, three

additional patients were found to have the deletion of 8 bp in enhancer II^[19]. This deletion was found mainly in sera from time points late after infection. The deletion of nt 1763-1770 was found to increase the ratio of pG to pC RNA, the replication of HBV *in vitro*, and to generate a new binding site for the transcription factor HNF-1^[12]. However, the deletion of nt 1763-1770 also deletes large parts of the binding site for COUP-TF, which was mapped to 1755-1768^[20-22]. We thus analysed whether enhanced replication from variant enhancer II/pC/pG promoter may also be caused by the deletion of the binding site for the repressor COUP-TF1. In contrast to previous studies we used HBV genome isolates with different genotype backgrounds and observed significant differences.

MATERIALS AND METHODS

Transfection and luciferase-assay

All experiments were performed with the differentiated human hepatoblastoma line HepG2^[23] cultivated in RPMI1640/10% FCS. The different expression constructs were used in luciferase assays. Briefly, $1-1.5 \times 10^6$ seeded cells were transfected with 1.5 µg luciferase construct and 3 µg of the COUP-TF1 or HNF-1 expression constructs using Lipofectamine (Life Technologies) according to the manufacturers description. All plasmids used were endotoxin free purified (EndoFree, Qiagen). After 48 h the transfected cells were harvested and lysed in Tris-buffer (250 mmol/L, pH 7.8) by 3 cycles of freezing and thawing. Protein concentration of lysates was determined by BCA-assay (Pierce). 15 µg of lysate protein adjusted to 50 µL with Tris-buffer were used in the luciferase activity assay^[24]. The results are shown as factors relative to basal luciferase expression and represent the mean values of three independent transfections.

Plasmids

All constructs for reporter assays were cloned into pLuci3 (Promega). The derived constructs were controlled by sequencing. pLEII-A-991 and pLEII-D-Ari: contain the enhancer II/core promoter sequence of genotype A (GenBank: X51970) or D (GenBank: Y07587) from nt 1400 to 1903 and have been described in^[25]. For pLEII-D-2.2.15 nt 1400 to 1903 were amplified from supernatants of the HBV expressing cell line HepG2.2.15^[26] and cloned accordingly. The sequence of this fragment was identical to GenBank U95551. HBV enhancer II, genotype D, with a corresponding truncated construct pL-EII (1730-1822). Derived from this sequence, constructs with an 8bp deletion of nt 1763-1770 or the frequently observed double mutation of 1762 (A/T), 1764 (G/T) have been cloned respectively (pL-EII(1730-1822)d8bp and pL-EII(1730-1822)A/T). For cotransfection expression vectors for HNF1^[27] or COUP-TF1^[28] were employed.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described by^[24] using nuclear extracts from HepG2 cells. For EMSAs the following oligonucleotides with the corresponding binding sites were used: wt (HBV-1755-1805-bs-s): 5'-GAT CCT TAG

GTT AAA GGT CTT TGT ATT AGG AGG CTG TAG GCA TAA ATT GGT CTG CGA-3'; wt (HBV-1755-1805-bs-as) 5'-GAT CTC GCA GAC CAA TTT ATG CCT ACA GCC TCC TAA TAC AAA GAC CTT TAA CCT AAG-3'; 8bp-Deletion (bs-HBVd8-1755-1805-s) 5'-GAT CTT AGG TTA AAT ATT AGG AGG CTG TAG GCA TAA ATT GGT CTG CGC A-3'; 8bp-Deletion (bs-HBVd8-1755-1805-as 5'-GAT CTG CGC AGA CCA ATT TAT GCC TAC AGC CTC CTA ATA TTT AAC CTA A-3'; 8bp-Deletion (HBVd8-dTBP-1755-1805-s) 5'-GAT CTT AGG TTA AAT ATT AGG AGG CTG TAG GCA TAA ATT GGT CTG CGC A-3'.

RESULTS

To analyse the effect of COUP-TF1 on a frequently found natural variant of the HBV EII/pC/pG promoter we cotransfected expression constructs for HNF1 and COUP-TF1 together with a luciferase reporter construct containing nt 1730-1817 (pL-pC-D-Ari) or nt 1730-1902 (pL-preG-D-Ari) (Figure 1A-C) into HepG2 cells as described^[25].

HNF1 showed a very weak transactivation of both promoter constructs irrespective if wt or the deletion construct were employed. COUP-TF1 showed a strong transactivation of all constructs. It appeared as if the transactivation was higher when constructs that contained the deletion of nt 1763-1770 were used. Two aspects of the latter results were surprising: COUP did not repress transcription from the wt-pG promoter as expected from previously published data^[13] and the deletion of a large part of the binding site for COUP in the Δ8bp-pG and -pC construct had no effect on the activation by COUP.

Our results for the pG and pC construct (nt 1730-1900) were seemingly in conflict with the data of^[13] who reported a repression of nt 1443 to 1990 of HBV by COUP. Thus, we cloned nt 1400 to 1902 into luciferase constructs. Figure 1C shows that COUP-TF1 also activated expression from the complete enhancer II of the isolates described in^[18,19,29]. No upstream element seemed to influence the activation of enhancer II by COUP-TF1 because all deletion constructs of pLEII-D-Ari and pLEII-D-Ari-Δ8bp were activated by COUP-TF1 (Figure 1).

Because pLEII-D-Ari was cloned from serum of patients infected during massive immune suppression and HBV genomes from these patients are known to contain many variants^[19], we analysed the effect of COUP-TF1 on enhancer II from an assumed wt-HBV genotype D genome. Figure 2 shows that this construct was also activated by COUP-TF1 even stronger than pLEII-D-Ari. In comparison we analysed a similar construct cloned from genotype A^[25]. Quite in contrast to the activation of pLEII-D-Ari, the genotype A construct pLEII-A991 was repressed by COUP-TF as reported for a construct containing enhancer II of HBV genotype C^[13].

COUP-TF1 is known to exert its repressive effect on HBV enhancer II of HBV genotype C through the NRRE around nucleotide 1755-1768^[13,14]. However, activation of HBV EII by COUP-TF1 also was observed when we tested constructs with a deletion of nt 1763-1770.

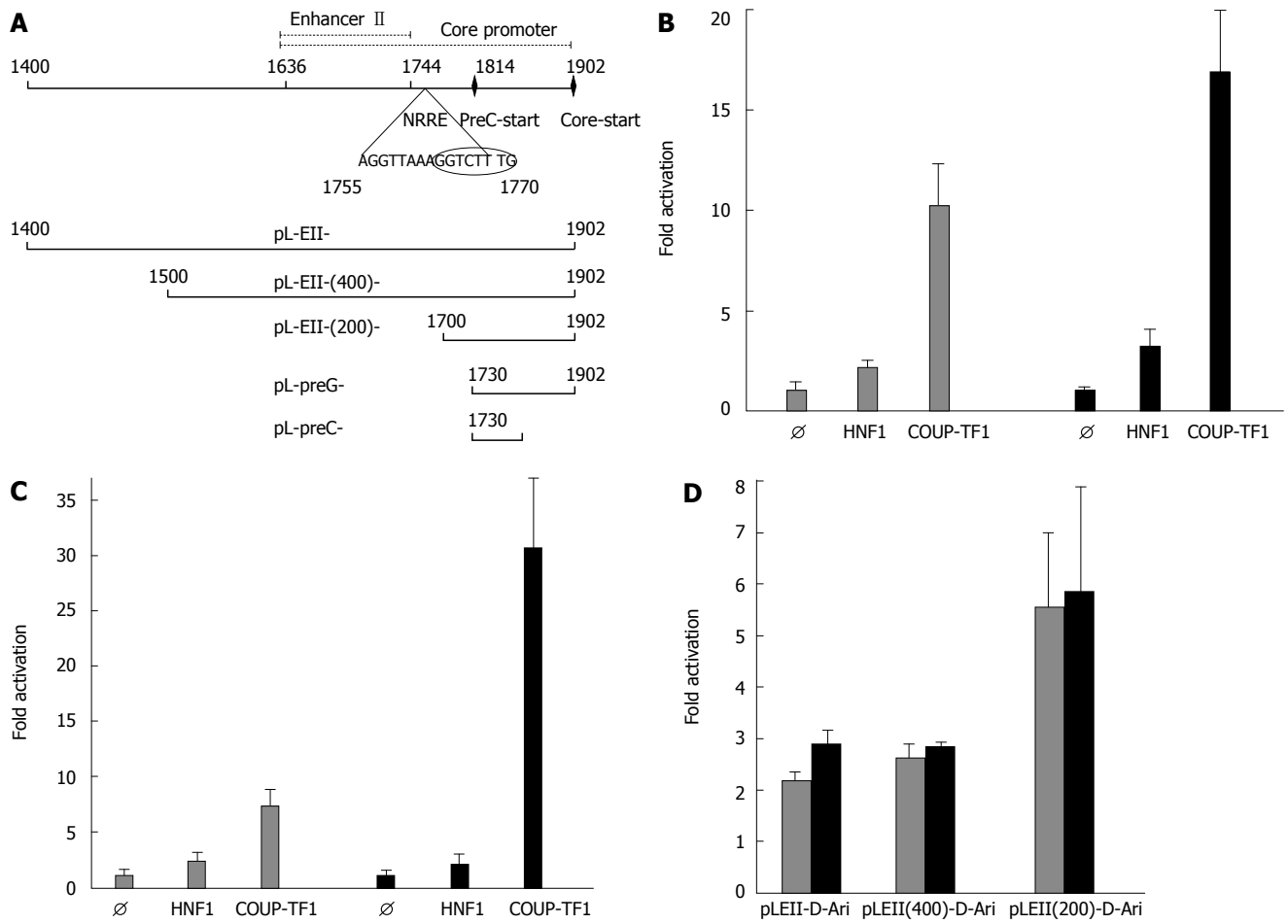


Figure 1 Structure (A) and activation of the precore (B), the pregenomic (C) and enhancer II (D) of HBV genotype D by COUP-TF1. A: Schematic structure of HBV enhancer II and the core promoter. The sequence of the nuclear receptor response element (NRRE) and the natural deletion of nt 1763-1770 (encircled) is indicated. Below the transcriptional elements the HBV fragments cloned into the luciferase reporter vector pGL3 are shown. For cloning the indicated nucleotides from two plasmids containing wt (GenBank: Y07587) and HBV with a deletion of nt 1763-1770^[18] were amplified and cloned. HepG2 cells were transfected with luciferase reporter constructs containing pregenomic promoter (pL-preG-D-Ari, wt in grey); B: Precore promoter (pL-preC-D-Ari, wt in grey); C: The complete and truncated wt enhancer II (pLEII-D-Ari, in grey) corresponding variant constructs with a deletion of nt 1763-1770 (Δ 8bp) in black. For cotransfection the empty expression vector (\emptyset) or expression vectors for HNF1^[27] or COUP-TF1^[28] were employed.

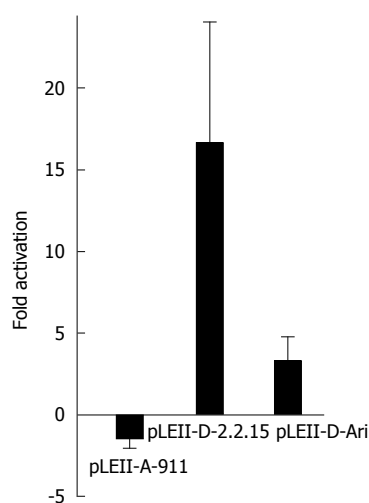


Figure 2 Influence of the nuclear receptor COUP-TF1 on enhancer II/core promoter constructs of different HBV genotypes. HepG2 cells were transfected with luciferase reporter constructs for the complete enhancer II (1400-1902) of genotype A (pLEII-A-911) or two constructs (pLEII-D-Ari and pLEII-D-2.2.15) derived from genotype D and the expression vector for COUP-TF1.

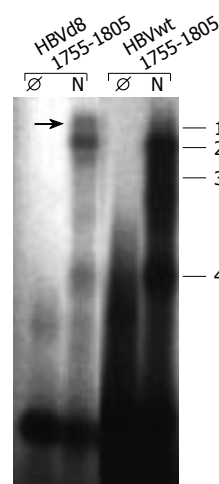


Figure 3 Deletion of nt 1763-1770 leads to altered band shifts in electrophoretic mobility shift assay with oligonucleotides nt 1755-1805. EMSA was performed as described by^[25] with oligonucleotide nt 1755-1805 wt (HBVwt 1755-1805) and with a deletion of nt 1763-1770 (HBVd8 1755-1805) incubated with (N) or without (\emptyset) nuclear extract prepared from HepG2 cells with a method^[25].

Thus, activation by COUP-TF1 may be exerted through other binding sites. To study the binding of the NRRE by cellular proteins we performed EMSAs with nuclear extracts prepared from HepG2 cells as described in^[24]. Figure 3 shows that the wild type oligonucleotide was bound

by several proteins purified from nuclear extracts of the human hepatocyte line HepG2. Four bands could be discerned in the EMSA with nuclear extract. Band 2 and 4 were common to wt and the 8 bp deletion. Band 1 was unique to the variant, band 3 to the wt.

DISCUSSION

Our results show that the ubiquitous transcription factor COUP-TF1 was a strong activator of wt and variant pG, pC promoter and enhancer II constructs (Figure 1 and 2) derived from sera of a single source outbreak^[18,19,29]. This result was surprising because Yu *et al.*^[13] reported that the wt-enhancer II (nt 1443 to 1990) was repressed by COUP-TF1. The involvement of distal elements in enhancer II that may be needed for activation by COUP-TF1 is unlikely because we did not observe a change in activation by deletion of upstream sequences (Figure 1D).

The NRRE seems to be dispensable for activation by COUP-TF1 in our genotype D constructs because all constructs containing a deletion of nt 1763-1770 were activated by COUP-TF1 as well as the wt-constructs (Figure 1). In addition, no binding of COUP-TF1 was observed by EMSA when we used an oligonucleotide containing a frequently found deletion of nt 1763-1770 (data not shown). These data support the findings of Yu and Mertz who reported that two frequently occurring natural point mutations of nt 1764 and 1766 also abolish binding by COUP-TF1^[14].

Our data are compatible with the report from the group of Mertz if HBV genotype differences are taken into consideration. Yu *et al.*^[13] found that the NRRE from nt 1755-1768 was essential for the repression of transcription from HBV enhancer II, pC and pG promoter of genotype C^[13,14]. We have analysed activation of HBV enhancer II, pC and pG promoter of genotype D by COUP-TF1. For activation by COUP-TF1 nt 1763 to 1770 of the NRRE seem to be dispensable. Thus, our data imply that activation of HBV enhancer II, pC and pG promoter is regulated by other elements than the NRRE. We can not rule out the possibility that COUP-TF1 exerts its activation of enhancer II of HBV genotype D by indirect mechanisms not involving direct binding to the promoter as in the activation of the vHNF1 promoter^[30].

Further analyses with constructs containing HBV DNA from other sources showed that very similar constructs of HBV enhancer II reacted differently to COUP-TF1: constructs derived from genotype A (Figure 3) or C^[13] were repressed by COUP-TF1 whereas constructs of genotype D derived from two different sera were activated. A sequence analysis (data not shown) of these four constructs revealed no differences in the NRRE at bp 1755 – 1768, which has been shown to be essential for repression by COUP-TF1^[13,14]. However, the sequences upstream of the NRRE showed sequence variability as expected for HBV isolates of different genotypes. However, we were not able to detect a sequence motif that may be indicative for activation or repression by COUP-TF1.

HBV genotypes influence the course and outcome of preventive and therapeutic measures^[8-10,31-33]. Very limited data are available on the effect of the sequence variability on *in vitro* properties of HBV, which may explain the different outcome of HBV infections depending on the genotype of HBV. In a relatively large analysis using HBV constructs of HBV genotypes A, C, D and E, Sommer *et al.* observed differences in splicing of the HBV pregenome^[34]. Other groups observed higher repression of

apoptosis by HBx derived from genotypes D compared to HBx of genotype C origin^[35]. Using another system, HBx of genotype D also showed a higher activity than HBx of genotype A in the induction of apoptosis^[36].

Thus, the variability induced by HBV genotypes may result in different molecular biology of HBV. However, there is more research to be done because our current results do not answer the question whether the differences described by several groups can be attributed to properties conserved in a given genotype or only represent certain variants that may occur in all 8 HBV genotypes.

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Necrosis of a large hepatic tumor after hemorrhage and subsequent selective arterial embolization

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Abstract

This case report describes a young female patient presenting with acute intra-abdominal hemorrhage originating from a large tumor in the liver, most likely a hepatocellular adenoma. The bleeding was stopped by selective embolization of right hepatic artery branches. Subsequently, partial hepatectomy was performed after 6 mo. Macro- and microscopic examination showed complete necrosis and absence of tumorous tissue. The patient was discharged without complications, and subsequent follow-up until 22 mo after resection did not reveal any new lesions in the liver. This case emphasizes the significance of selective arterial embolization in the management of bleeding liver tumors and questions the need for (partial) hepatectomy after this procedure in selective cases.

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Key words: Liver hemorrhage; Selective arterial embolization; Hepatocellular adenoma

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INTRODUCTION

Hepatocellular adenomas are uncommon benign liver tumours that are present mainly in women of reproductive age^[1]. Their existence is associated with the use of oral

contraceptives^[2]. The first clinical presentation of the tumour can consist of abdominal pain, hepatomegaly, or hepatic (subcapsular) hemorrhage and shock^[1]. For the latter presentation, emergency surgery by partial hepatectomy is the current treatment of choice. However, this treatment is associated with high morbidity and mortality rates^[3,4]. Therefore, several groups suggest a role for initial conservative treatment^[1,5].

Selective arterial embolization is a novel method for the management of intra-abdominal hemorrhage without the need for major surgery, and our hospital has gained considerable experience with this procedure. However, the use of this elegant method when coping with liver hemorrhage has only been described incidentally^[1,5,6].

After any initial emergency treatment, secondary partial hepatectomy is generally recommended in patients with lesions ≥ 5 cm, because of increased risk of rupture as well as malignant degeneration^[1,5]. The case we present, however, suggests a role of selective arterial embolization not only in the primary emergency situation, but also as a therapeutic tool to achieve necrosis of an arterially vascularized liver tumor.

CASE REPORT

A 35-year old woman was referred to our hospital with a two-day history of right abdominal pain without any previous trauma. The patient had no medical history, her medication consisted of oral contraceptive treatment only (30 μ g ethinylestradiol, 150 μ g levonorgestrel, taken during eleven years). Despite initial fluid supplementation, her heart rate was elevated (110/min) with normal blood pressure. Further physical examination showed tenderness of the right upper abdomen. Hemoglobin levels were low (78 g/L). INR was 1.0, APTT 21.5 s, PTT 12.5 s and thrombocytes 213×10^9 /L. Serum liver enzyme levels were elevated (ASAT 550 U/L, ALAT 607 U/L, Alk.Fos 75 U/L, γ GT 109 U/L, LDH 758 U/L). She was transfused with 2 units of red blood cells. Computed tomography (CT) scan of the abdomen revealed a large subcapsular hematoma surrounding a tumorous mass in segment IV-VII of the liver, as well as intraperitoneal fluid. Arteriography confirmed hemorrhage originating from the right hepatic artery, which was also responsible for the vascularization of the tumor. Subsequently, selective arterial embolization of the majority of right hepatic artery branches was performed in order to stop the bleeding using both polyvinyl alcohol (PVA) particles and a mixture of enbucilate (histoacryl) and

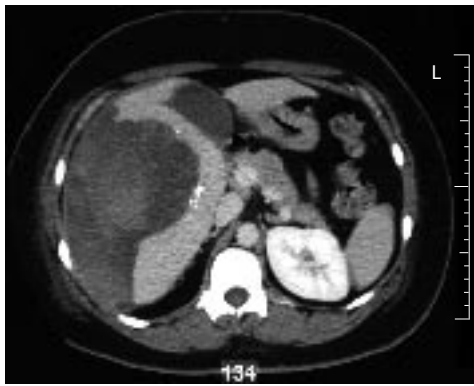


Figure 1 Contrast-enhanced computed tomography (CT) scan of the abdomen 6 d after selective arterial embolization, showing liver hemorrhage with a circumscribed mass as its conceivable origin.



Figure 2 Arterial phase CT scan of the abdomen before partial hepatectomy showing remains of the hematoma and the known circumscribed mass, which is not vascularized arterially. Note the arterial deposition of histoacryl and lipiodol along the tumor as a result of selective embolization.



Figure 3 Macroscopic view of the resected tumor (7 cm) consisting entirely of necrotic material.

iodized poppy-seed oil (lipiodol). Contrast-enhanced CT scan 6 d after embolization clearly showed a circumscribed tumorous mass (7 cm) in segment V of the liver, most likely a hepatocellular adenoma (Figure 1). Further hospital stay was unremarkable and the patient was discharged 11 d after admission. Following our department's protocol on liver adenomas with a diameter ≥ 5 cm, elective resection of segment V was performed six months later. Preoperative contrast-enhanced CT scan showed the remains of both the hematoma and the known circumscribed mass (Figure 2). Arterial perfusion of segment V of the liver was absent whereas portal perfusion was intact. The resected tumor consisted completely of necrotic material (Figure 3). No clear tumor classification could be made by histological examination due to absence of tumorous material, but the macroscopic signs of necrosis were confirmed. Tumor markers (CEA, CA 19.9 and α -fetoprotein) were in the normal range. The patient was discharged without complications, and subsequent follow-up until 22 mo after resection did not reveal any new lesions in the liver. She was advised to refrain from oral contraceptive medication.

DISCUSSION

The increased chance of rupture of large hepatocellular adenomas makes the presence of such a tumour a

considerable potential hazard. Management of this condition still needs improvement. The presence of a ruptured hepatocellular adenoma cannot be proven by CT scan alone, but is strongly suggested by our patient's clinical presentation. Although hepatocellular carcinoma and focal nodular hyperplasia lesions have also been described as causes of liver hemorrhage^[7], their presence is unlikely in this patient. The presence of liver adenomas in young women is associated with the use of oral contraceptives. In this case no specific histologic tumor classification could be made, since cessation of arterial blood flow by embolization caused necrosis of the tumor. This corresponds with the fact that hepatocellular adenomas are only vascularized arterially. It has to be noted that regression of liver adenomas is possible not only after ischemia, but also after hormone withdrawal^[8], hemodialysis^[9] and dietary therapy for glycogen storage disease^[10].

Embolization is safe and successful in stopping the hemorrhage. The necrosis of the tumor may point to a future role of selective arterial embolization in the management of either bleeding or non-bleeding hepatocellular adenomas. Furthermore, this case argues in favour of conservative follow-up after embolization, regardless of the initial size of the adenoma. This would further limit the indications for surgery in a selected group of patients, resulting in reduction of patient morbidity and mortality.

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CASE REPORT

Biliary cystadenoma with mesenchymal stroma: Report of a case and review of the literature

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Abstract

Biliary cystadenomas are rare, cystic neoplasms of the biliary ductal system that usually occur in middle-aged women. They cannot be safely differentiated from cystadenocarcinomas before operation and should always be considered for resection. Cystadenomas have a strong tendency to recur, particularly following incomplete excision, and a potential of malignant transformation. Therefore, complete resection is the therapy of choice and thorough histopathologic evaluation is imperative. A case of benign biliary cystadenoma with mesenchymal stroma is presented along with a review of the relative literature addressing the clinical presentation, histology, histogenesis, differential diagnosis, imaging features, treatment and prognosis of this interesting and rare entity.

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Key words: Biliary cystadenoma; Biliary cystadenocarcinoma; Mesenchymal stroma

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INTRODUCTION

Biliary cystadenomas are rare, benign but potentially malignant, multilocular, cystic neoplasms of the biliary ductal system^[1,2]. They usually arise in the liver (80%-85%)^[2,3], less frequently in the extrahepatic bile ducts^[2,3] and rarely in the gallbladder^[4], accounting for less than 5% of cystic

neoplasms of the liver^[5,6]. Less than 100 reports of intrahepatic biliary cystadenomas are identified in the medical literature^[5,7-10].

They more frequently occur in middle-aged women^[7,10,11]. The patients may be asymptomatic with their tumors discovered incidentally during radiographic evaluation or surgical exploration for other clinical indications^[6]. However, they often have vague abdominal complaints related to extrinsic compression of the stomach, duodenum, or biliary tree^[5,7,12]. The premalignant nature or potential for malignant transformation or degeneration and the tendency to recur, particularly when treated with techniques other than complete excision, are of great concern in these tumors^[7,10,11]. Furthermore, since clinical presentation as well as laboratory and imaging data are highly variable and non specific, cystadenoma cannot be differentiated preoperatively from cystadenocarcinoma^[1,5,6,10,13].

Such entities in surgical pathology are presented mainly as case reports or retrospective collective studies since their rarity prohibits a prospective evaluation. A case of benign biliary cystadenoma with mesenchymal stroma is presented here along with a literature review.

CASE REPORT

A 39-year old woman complained of vague abdominal pain and increasing abdominal girth. No other signs or symptoms were discovered on clinical examination apart from marked hepatomegaly. All blood tests were normal including liver function tests and levels of serum neoplastic markers such as carbohydrate antigen 19-9 (CA 19-9), carcinoembryonic antigen (CEA), carbohydrate antigen 125 (CA 125), and α -fetoprotein (AFP). Anti-echinococcal IgM and IgG antibodies and viral markers for hepatitis B and C were negative. Ultrasound (US) finding of a cystic lesion of the liver led to a computed tomography (CT) scan that revealed a large (19 cm \times 16 cm \times 10 cm), well-encapsulated, binodular, cystic formation of the liver occupying most of the right lobe (Figure 1). The lesion appeared to exceed the limits of the liver, reaching the right iliac fossa. A radiological diagnosis of hydatid cyst was offered.

The patient underwent laparotomy under an extended right subcostal incision that revealed a large cystic formation with no macroscopic resemblance to a hydatid cyst. A decision for right hepatic lobectomy was made and segments V-VIII were excised under proximal vascular control. No intraoperative blood transfusion was required. The postoperative course was uneventful and the patient

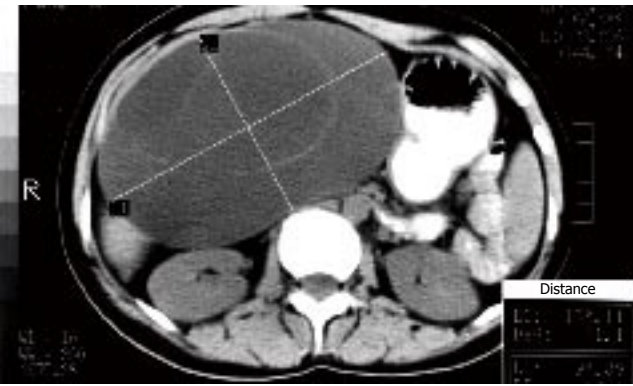


Figure 1 Abdominal CT scan revealing a large binodular cyst occupying most of the right liver lobe.

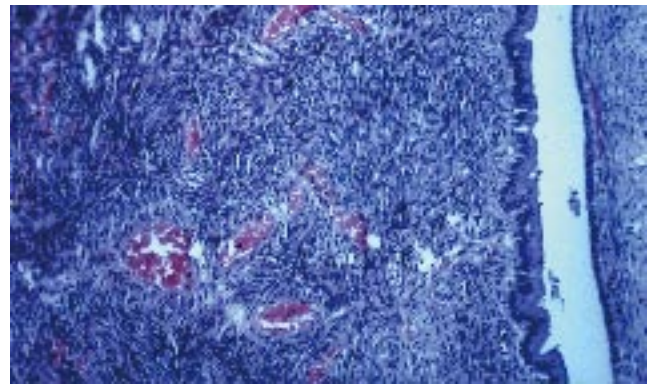


Figure 2 Histologic appearance of the simple columnar and cuboidal epithelium that is typical of biliary cystadenoma. The underlying mesenchymal stroma is densely cellular resembling ovarian.

was discharged six days after operation.

The cytology report of the cystic fluid identified clusters of glandular cells with round nuclei and clear cytoplasm. The most possible diagnosis was biliary cystadenoma. The final histology report referred to an 18 cm × 16 cm gray-white cystic lesion with 0.1-0.3 cm of wall thickness indicating intramural microcystic formation. The lesion was characterized as a hepatobiliary cystadenoma. The epithelium was simple columnar and cuboidal with no atypia and had an underlying densely cellular mesenchymal stroma resembling ovarian (Figure 2). Histological examination showed complete removal of a benign hepatobiliary cystadenoma with mesenchymal stroma.

The patient has remained free of disease for a period of 4 years on repeated CT scans since she was discharged.

DISCUSSION

Cystadenomas are rare, benign but potentially malignant, multilocular, cystic neoplasms of the biliary ductal system^[1,2,7,10], accounting for less than 5% of cystic neoplasms of the liver^[5,6,10]. The incidence of intrahepatic biliary cystadenomas is between one in 20 000 to one in 100 000 people while the incidence of cystadenocarcinomas approximately one per 10 million patients^[14]. Hepatobiliary cystadenomas can occur at any age^[15], but they are usually seen in middle-aged women^[7,10,11,16]. Approximately 85%-95% of the patients are women^[12,15,17]. Even though they are true proliferative epithelial tumors, their progression is characteristically slow and years are required before they enlarge^[11]. Their size is variable ranging from 1.5 to 30 cm in diameter^[2,5,11,16,18]. They usually arise in liver (80%-85%)^[2,3,7,10,19,20], less frequently in extrahepatic bile ducts^[2,3,7,10,17,19] and rarely in gallbladder^[4]. Approximately 50%-55% of cystadenomas are located in the right lobe with the remaining located in the left lobe or in both lobes (30%-40% in the left lobe and about 15%-20% in both lobes) while few arise from extrahepatic ducts^[2,11,12,21]. Accordingly, the common or the right bile duct can be identified as the origin in more than half of cases.

Although reports of cystadenomas have been increasing due to advances in imaging diagnoses and the

common use of US and CT, less than 100 reports of intrahepatic biliary cystadenomas are identified in the literature^[5,7-10,22] and only 26 extrahepatic cystadenomas have been reported^[17,23]. A review of the literature is provided along with a discussion addressing the clinical presentation, histology, histogenesis, differential diagnosis, imaging features, treatment and prognosis for this rare entity.

Clinical presentation

The patients may be asymptomatic with their tumor discovered incidentally during radiographic evaluation or surgical exploration for other clinical indications or even at autopsy^[6,24]. However, they often have vague abdominal complaints related to extrinsic compression of adjacent structures such as the stomach, duodenum, or biliary tree. Symptomatic patients present with an insidious onset of symptoms due to the slowly growing nature of the neoplasm^[12].

The typical patient is a white female presenting with abdominal discomfort, swelling, gradual increase in abdominal girth and/or pain and a palpable abdominal mass^[5,7,11,12]. Right upper quadrant or epigastric pain along with increasing abdominal girth or awareness of an abdominal mass are the main complaints in about 60% of the patients as reviews of the reported cases have shown^[21]. In another series, complaints include abdominal pain in 74%, abdominal distension in 26%, and nausea/vomiting in 11% of the patients^[25]. The patients may less frequently have gastrointestinal obstruction leading to nausea and vomiting, dyspepsia, anorexia, weight loss or ascites^[11,26].

When complicated, symptoms include biliary obstruction^[5,7,12], rupture^[19], bacterial infection^[19], intracystic hemorrhage^[19,22] or malignant transformation^[7,10]. Biliary obstruction may be either due to the tumor itself^[21,27] or either, rarely, due to the secretion of mucin^[20,28] and is manifested as jaundice, biliary colic, cholangitis, nausea, fever, chills, itching, or steatorrhea^[22]. Such cases represent approximately 35% of the patients with cystadenomas^[24]. Patients may experience intermittent jaundice or repetitive episodes of biliary colic or cholangitis for a long period of time before the diagnosis^[21,22]. Obstructive jaundice,

although not always present^[29], is the most frequent presenting symptom in patients with extrahepatic cystadenomas^[17,19]. Other reported symptoms are right upper quadrant or epigastric pain, nausea, vomiting, skin itching and occasional diarrhea^[29]. On the contrary, in intrahepatic cystadenomas, biliary obstruction is rarely the chief presenting complaint of the patients^[22]. Any patient with clinical or biochemical obstructive jaundice of unknown etiology or recurrence of a liver cyst following surgical treatment should be suspected of having an intrahepatic or extrahepatic cystadenoma^[28-30] or a cystadenocarcinoma^[5,10].

Histology

Biliary cystadenomas are usually large, multiloculated, with internal septation and nodularity and surrounded by a dense cellular fibrostroma. Although it may be extremely rarely be unilocular^[18,22], multilocularity of the tumor is a key feature that distinguishes cystadenomas from developmental cysts^[16]. Biliary cystadenomas are usually globular, with a smooth external surface and a smooth or trabeculated inner surface and contain locules of variable sizes^[10,16]. The internal surface of the cyst may also have papillary infoldings or smaller cysts^[10]. On light microscopy, cystadenoma consists of three layers: a cyst lining of biliary-type epithelium, a moderately-to-densely cellular stroma, and a dense layer of collagenous connective tissue^[31].

The cyst wall consists of a single layer which is typical of biliary-type epithelium containing cuboidal-to-tall columnar and nonciliated mucin secreting epithelial cells with papillary progressions, pale eosinophilic cytoplasm and basally oriented nuclei. The cyst usually contains clear mucinous fluid^[10,19] which raises the possibility of a malignant component when hemorrhage occurs^[4,11], unless there is a trauma history. Rarely, the fluid within the cyst may be bilious, purulent, proteinaceous, gelatinous, clear or mixed. The septa of the tumor may show calcification.

Foci of epithelial atypia or dysplasia consisting of nuclear enlargement and hyperchromasia, multilayer, loss of polarity, and mitotic activity indicate potential malignant changes^[7,16]. Anaplasia or pleomorphism, severe architectural atypia (such as exophytic papillae) and capsular or stromal invasion are features of malignancy^[5,10,16]. The premalignant progression is usually based on the histologic presence of intestinal metaplasia which is characterized by the presence of numerous goblet cells^[7]. Careful pathologic evaluation is emphasized since malignant degeneration or transformation can be detected only after thorough sectioning. Histological differentiation of biliary cystadenoma from other cystic liver lesions is usually based on its multilocularity, columnar epithelium, papillary infoldings, and ovarian-like stroma^[16].

Similar lesions occur in the pancreas and ovary. Two histological variants of biliary cystadenoma are recognized, a serous type and a far more common mucinous type^[16]. The rare serous variety resembles serous cystadenoma of the pancreas and is not known to undergo malignant transformation^[16]. The mucinous type is predominantly a tumor of middle-aged women and is similar to mucinous

cystadenoma of the pancreas^[16].

If the underlying subepithelial stroma is densely cellular resembling ovarian stroma as in our case, it is referred as "mesenchymal stroma"^[5,8]. The stromal cells are spindle-shaped and usually immunoreactive with vimentin, alpha-smooth muscle actin, and muscle-specific actin and less frequently with desmin, estrogen and progesterone receptors^[8,16,27]. Although mesenchyme of these tumors resembles ovarian stroma morphologically, such immunohistochemical features are characteristic of myofibroblasts^[8,31]. Mesenchymal stroma is more often observed in the mucinous type of cystadenoma^[16]. There are two distinct types of cystadenomas based on the presence or absence of "mesenchymal stroma"^[5]. Cystadenoma with mesenchymal stroma occurs exclusively in women^[5,7,8,31,32] while cystadenoma without mesenchymal but with hyaline stroma arises in both men and women and tends to occur in older patients^[5,32]. Cystadenoma with mesenchymal stroma is regarded as a precancerous lesion^[33] but the type without mesenchymal stroma seems to undergo malignant degeneration much more frequently^[32]. Patients with cystadenocarcinoma with mesenchymal stroma have a good prognosis whereas the prognosis of patients with cystadenocarcinoma without mesenchymal stroma is poor, especially in men^[7]. The malignancy arises from the epithelial component in most cases^[33], although sarcomatous transformation of the mesenchymal stroma has been reported^[31,34].

Etiology-histogenesis

The origin of biliary cystadenomas is unclear. Theories on their etiology and histogenesis are not solid since both acquired and congenital origins have been proposed. Experimental studies by Cruickshank *et al*^[34] may support the theory of an acquired lesion. The development of the tumor as a reactive process to some focal injury has also been mentioned^[10]. Mesenchymal stroma, though resembling ovarian microscopically, is more akin to the primitive mesenchyme in embryonic gallbladder and large bile ducts^[8,9], suggesting that the tumor may arise from ectopic embryonal tissue destined to form the gallbladder^[9] or from ectopic embryonic rests of primitive foregut sequestered within the liver^[5,10,33]. Demonstration of endocrine cells in about 50% of hepatobiliary cystadenomas and cystadenocarcinomas could also suggest an origin from intrahepatic peribiliary glands^[35]. The presence of hamartomatous structures and development abnormalities supports the theory of congenital origin in at least some cystadenomas^[10,36].

A possible hormonal dependance of cystadenomas with mesenchymal stroma has also been proposed since immunohistochemical studies showing the characteristics of mesenchymal stromal cells, have revealed myofibroblastic phenotype and expression of progesterone and estrogen receptors^[27,37,38]. In addition, there are reports of this tumor occurring in oral contraceptive users, suggesting that estrogen-containing oral contraceptives may serve as tumor promoters^[11,38]. Ectopic ovarian tissue, however, is considered an unlikely origin of these tumors^[5,8].

It is uncertain whether biliary cystadenocarcinomas are *de-novo* cancer or are derived from cystadenomas. They are generally thought, however, to arise from preexisting benign cystadenomas since many cystadenocarcinomas contain areas of cystadenoma in the same sample^[5,10,33,39].

Differential diagnosis

In the evaluation of cystic hepatic lesions, a high index of suspicion is imperative. Moreover, since these neoplasms have a strong tendency to recur and undergo malignant transformation, differentiating between cystadenomas and other cystic liver lesions is substantially important. Differential diagnosis includes simple liver cysts, parasitic cysts (particularly hydatid cysts), haematomas or post-traumatic cysts, liver abscesses, congenital cysts, polycystic disease, hamartomas, Caroli's disease, and neoplastic lesions such as biliary cystadenocarcinoma, undifferentiated embryonal sarcoma, cystic metastasis, metastatic pancreatic or ovarian cystadenocarcinoma, biliary papilloma, cystic primary hepatocellular carcinoma, cystic cholangiocarcinoma, and hepatobiliary mesenchymal tumors (particularly biliary smooth muscle neoplasms) such as biliary leiomyoma, adenomyoma, and primary hepatic leiomyosarcoma^[2,27,40]. Preoperative and intraoperative diagnosis of biliary cystadenomas and cystadenocarcinomas can be very difficult and differentiation between these two entities can be safely done only after histopathologic evaluation^[1,5,6,10,33,40].

Specific attention should be paid to liver hydatid disease, especially in countries with a high incidence of the disease. Since its imaging features are similar to those of cystadenoma as in our patient, preoperative differentiation may be impossible without serologic tests^[2,24,41]. Anti-echinococcus granulosis and anti-amoebic serologic tests, estimation of CA 19-9, CEA and AFP levels, general evaluation of liver and renal function as well as abdominal US, CT and magnetic resonance imaging (MRI) should be performed. Liver function tests may be normal or elevated in cases of intrahepatic or extrahepatic biliary duct compression^[11,27,29].

Although they do not rule out cystadenoma when normal^[14], serum CA 19-9 levels are believed to be a valuable marker in the diagnosis and monitoring in the postoperative follow-up since they are reported to return to normal after complete resection^[11,42]. Nevertheless, immunoreactivity for CA 19-9 is lost therefore minimizing the possible benefit of serial follow-up when cystadenoma is transformed to cystadenocarcinoma^[43].

Measurement of cyst fluid CA 19-9 and CEA levels has been advocated as an adjunctive preoperative procedure for enhancing the accuracy of differentiation of cystadenomas and cystadenocarcinomas from other hepatic lesions^[14,44]. High levels of CA 19-9 and CEA can be encountered in cystic fluid or in epithelial lining of biliary cystadenomas, particularly in those with mesenchymal stroma^[5,14,30,41,44] and cystadenocarcinomas^[45]. These elevated cyst fluid tumor markers clearly indicate the neoplastic features and biliary origin of these cysts^[5]. Elevation of CEA may, however, be moderate or not always present^[14]. On the contrary, substantially higher levels of CA 19-9 in

cystic fluid are observed in the majority of cystadenomas compared with those in simple cysts, echinococcal cysts, and polycystic liver disease^[14,30,41,42,44]. Therefore, though not allowing differentiation between cystadenoma and cystadenocarcinoma, measurement of CA 19-9 in cystic fluid obtained by fine needle aspiration as well as in serum may be helpful in the differential diagnosis of hepatic cystic lesions. Moreover, Koffron *et al.*^[14] have proposed a diagnostic and therapeutic algorithm for hepatic cysts and particularly intrahepatic biliary cystadenomas based on CA 19-9 and CEA levels and cytology along with laparoscopic cyst wall biopsy.

Percutaneous fine needle aspiration also provides fluid for bilirubin concentration analysis, which is suggestive of communication of cystadenoma with the biliary tract when it is elevated^[14], and for cytologic evaluation which may be helpful in excluding hepatic abscess, cystic metastases and other cystic lesions. Although there are reports of cystadenocarcinomas arising from cystadenomas diagnosed by percutaneous fine needle aspiration cytology^[45,46], it is not very accurate since this procedure relying on adequate sampling may miss the microscopic foci of the carcinoma in cystadenoma^[44,47,48]. Thus, correlation of aspiration cytology with clinical and radiological data has been suggested^[14,47]. Fine needle aspiration and needle biopsy for diagnosis, however, may also risk dissemination of tumor cells and is not generally recommended, particularly when surgery is planned^[30,48]. Pleural and peritoneal dissemination of tumor cells caused by aspiration has been reported^[48,49].

Furthermore, examination of epithelial cells of several types of hepatic cysts by mucin histochemistry and immunohistochemistry reveals different features of these cells regarding mucus and antigenic expression among the hepatic cysts^[50]. Epithelial cells of non-parasitic simple cysts and adult-type polycystic liver show similar mucin-histochemical and immunohistochemical features, and are characterized by little mucin and weak immunoreactivities to several antibodies. On the contrary, epithelial cells of cystadenoma and cystadenocarcinoma are characterized by much mucin and moderate to strong immunoreactivities to cytokeratins CAM5.2 and AE1 and AE3 as well as to CA 19-9, CEA and epithelial membrane antigen (EMA)^[16,50].

Although frozen sections may be helpful in differentiation of cystadenomas and cystadenocarcinomas from other cystic hepatic lesions^[18], they are not very useful due to the variability in histology of cystadenomas and their inability to rule out cystadenocarcinomas^[48,51,52]. Careful histopathologic evaluation of the resected specimen, therefore, constitutes the only safe diagnostic modality of hepatobiliary cystadenomas and cystadenocarcinomas while malignant degeneration or transformation of a cystadenoma can only be detected after thorough sectioning.

Imaging features

Diagnosis and differential diagnosis of cystadenoma from other cystic hepatic lesions are mainly based on abdominal US, CT scan and MRI. Preoperative US assessment is almost always performed but cannot replace the

diagnostic value of CT. Moreover, the role of US and CT is considered complementary^[53]. By US the tumor appears well-demarcated, thick-walled, noncalcified, anechoic or hypoechoic, globular or ovoid, cystic mass and may present thin internal septations, which are highly echogenic, mural nodules and polypoid or papillary infoldings^[2,11,15,24,53]. Dilatation of intrahepatic or extrahepatic bile ducts may also be disclosed^[27]. In case of intracystic hemorrhage, US may show a hyperechoic mass with no septation^[22].

The need to determine the size, morphology and anatomic relation to surrounding structures, particularly major vessels of the lesion prior to intervention is crucial and CT is of great help for the surgeon. Additionally, CT during arteriography is useful to demonstrate the tumor vascularity^[45]. Common features on CT scan include low-density, well-defined, lobulated, multilocular, thick-walled, cystic masses with internal septa and occasionally mural nodules^[2,12,15,26,53]. Intravenous contrast on CT enhances the cyst wall and septations. CT may sometimes demonstrate dilatation of intrahepatic or extrahepatic bile ducts. Rarely capsular, mural or septal calcification presents on US or CT^[2,15,54]. Although such features can be identified with CT and are essential for differentiating cystadenoma from other cystic liver lesions, a possible preoperative diagnosis is rarely suspected due to the rarity of the disease.

Demonstration of communication between the tumor and the biliary tract is of important diagnostic value both in identifying the site of origin of the tumor and in differentiating biliary cystadenoma/cystadenocarcinoma from other hepatic cystic lesions^[54]. Such a communication can be demonstrated with percutaneous transhepatic cholangiography or cystography (PTC), endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance cholangiopancreatography (MRCP), intraoperative cholangiography, cyst fluid examination for bile content, or surgical exploration^[12,14,18,45,54,55]. Moreover, since the potential for cystadenomas to extend intraluminally into extrahepatic ducts has been described^[41], preoperative extrahepatic biliary imaging is imperative when surgical management is planned. It is noteworthy that no biliary connection has been identified, even at the time of surgery, in the majority of reported cystadenomas^[11]. In a review of the literature, Sato *et al*^[45] reported that such a biliary fistula was found in only 21 cystadenomas and 16 cystadenocarcinomas.

ERCP or PTC may show an intraluminal filling defect that may be the tumor or represent intraluminal mucin^[20]. An infrequent finding is the endoscopic visualization of mucin being extruded through the ampulla of Vater^[18]. PTC can provide further information on biochemical and cytologic examinations of the cyst fluid^[45]. However, fine needle aspiration cytology is not very accurate^[44,47,48] while aspiration may risk possible tumor pleural or peritoneal seeding^[48,49]. Therefore, it should be avoided in cases of obvious or suspected cystadenocarcinoma, especially when surgery is planned^[48]. ERCP may also be performed in order to reveal or exclude compression or displacement of intrahepatic and extrahepatic bile ducts and is especially important if patients present with jaundice^[19]. Furthermore, diagnosis of a cystadenoma of the

extrahepatic biliary tree can be obtained by intraoperative cholangiography and/or choledochoscopy, PTC, ERCP, MRCP or spiral CT scan^[21,55-57].

MRI is a valuable tool for the diagnosis and differentiation of cystadenoma from other cystic liver lesions while combination of MRI with MRCP is also even more useful^[55]. On T1-weighted images, MRI reveals a fluid-containing, multilocular, septated mass with homogenous low signal intensity, the wall and septa of which become enhanced after administration of Gd-DTPA^[2,54,55]. On T2-weighted images the fluid collections within the tumor demonstrate variable, homogenous high signal intensity while the wall of the mass is represented by a low-signal-intensity rim^[2,54,55]. Variable signal intensities on T1- and T2-weighted images depend on the presence of solid components, hemorrhage, and protein content^[2,54,55]. On T1-weighted images, the signal intensity may change from hypointense to hyperintense while septations may be obscured, and only mild enhancement of the cyst wall is noted after Gd-DTPA administration, as protein concentration and viscosity of the cyst fluid increase. In contrast, on T2-weighted images, signal intensity of the cyst fluid may decrease. Similar changes of the typical MRI appearance of cystadenoma may be caused by internal hemorrhage^[26]. MRI may disclose dilated intrahepatic or extrahepatic bile ducts or demonstrate the relationship of the lesion to vascular structures and, thus, may be helpful in planning the surgical procedure.

Preoperative assumption that the lesion is benign based on US, CT or MR findings is not safe and therefore not recommended^[48]. The presence of irregular thickness of the wall, mural nodules or papillary projections indicates the possibility of malignancy^[2,7,45,53]. Papillary projections in the cyst can be seen on contrast-enhanced CT, and are characteristic of malignant neoplasm^[4]. However, there are cases in which papillary projections are not shown clearly on CT. Hypervascularity of mural nodules on CT during arteriography may also indicate malignancy of the lesion^[45]. Septation without nodularity suggests the diagnosis of cystadenoma whereas septation with mural or septal nodules, papillary infoldings, discrete solid masses, and thick, coarse calcifications is suggestive of cystadenocarcinoma^[4,7,54]. Changes in appearance of the cyst wall may also suggest malignant transformation^[45]. Despite these features, however, imaging differentiation criteria between biliary cystadenoma and cystadenocarcinoma have not yet been established^[14,17,33].

Angiography is advisable and may be helpful both in clarifying the hepatic arterial anatomy and in differentiating cystadenocarcinoma from benign cystadenoma but seems not essential while the resectability of the tumor is assessed. Common findings include an avascular or hypovascular lesion with a faint rim of contrast-material accumulation in the wall and septa of the mass in the parenchymal phase and, occasionally, a tumor blush at the periphery corresponding to the papillary infoldings along with displacement of regional intrahepatic vessels^[1,11,12,22,26]. Signs raising the suspicion of malignancy are considered to be attenuated intracystic vessels, stretching of thin hepatic arteries, intracystic hypervascular mural nodules,

irregular calibers of the peripheral arteries in the arterial phase, and light stains in the parenchymal phase^[40,45]. Attenuated arteries and tumor stain at the central portion of an avascular or hypovascular lesion are the typical angiographic features of a cystadenocarcinoma^[40].

Although difficult, correct preoperative and/or intraoperative diagnosis of cystadenomas and cystadenocarcinomas is of utmost importance in planning the appropriate surgical procedure. Among all intraoperative diagnostic and surgical procedures intraoperative ultrasound is considered the most sensitive^[58]. Intraoperative US can be helpful for the diagnosis of biliary cystadenoma^[3,18] and determining its resectability^[11]. It may occasionally allow the surgeon to differentiate between the smooth wall of a cystadenoma and the infiltrative wall of a cystadenocarcinoma^[18]. However, it cannot usually confirm the relationship between the tumor and the bile system while it may be difficult to distinguish an extrahepatic cystadenoma from a pancreatic cystadenoma because of their similar appearance on US^[51]. In patients operated laparoscopically, laparoscopic ultrasonography has been reported as an essential supplement to inspection of the lesion via laparoscopy^[14].

Intraoperative cholangiography is also considered very helpful since it can demonstrate both intrahepatic^[12,18] and extrahepatic lesions^[23]. It may allow fluid aspiration for cytology^[18] and may reveal communication between the lesion and the biliary system^[18] although not always^[12]. In addition, it can be performed after the surgical procedure in order to exclude any bile duct injuries or retained cysts^[18]. It has been proposed that intraoperative cholangiography and/or choledochoscopy should be performed to diagnose extrahepatic cystadenomas^[23].

Treatment and prognosis

Resection is the management of choice for all multiloculated cystic hepatic lesions^[19,59]. If a cystadenoma is suspected or has been diagnosed, surgery is indicated even in asymptomatic patients, since cystadenoma and cystadenocarcinoma cannot be reliably differentiated on the basis of radiologic and macroscopic criteria^[1,5,6,10,33,48,54]. The extent of resection remains to be determined since partial resection with occasional ablation of the residual cyst using electrocautery or argon beam coagulation and/or omentopexy^[14,33,60], and lobectomy^[14,61] as well as wedge resection^[14] and enucleation^[14,19,50,62] have been reported.

In cases of communication of an intrahepatic cystadenoma with the biliary tract, biliary fistulae should be confirmed^[45]. When such fistulae are identified, resection of the tumor should be supplemented with suture closure of the fistulae^[14,45] or resection of the affected bile duct and bilioenteric reconstruction particularly if postoperative leak or intrahepatic biliary obstruction after suture control is concerned^[14,41]. In treatment of extrahepatic cystadenomas, resection of the tumor should be supplemented with resection of the affected bile duct and bilioenteric anastomosis^[17]. In cases of compression of intrahepatic or extrahepatic ducts, postoperative cholangiography may be performed to identify resolution of biliary compression^[45].

Incidental finding of a cystadenoma during surgery

for other clinical indications demands a complete surgical resection of the tumor^[6]. Although incidental finding of a cystadenoma after open or laparoscopic fenestration of a hepatic cyst also requires complete resection, it has been proposed that after complete enucleation of the cyst, strict follow-up could be considered as the definitive treatment, demanding the surgical intervention only in case of recurrence or high suspicion of malignancy^[6,63]. However, recurrence of symptoms 8 and 18 months after laparoscopic fenestration of an unsuspected cystadenoma has been reported^[64].

Total tumor extirpation with a wide margin of normal liver provides a chance for cure^[5,7,10,65]. Techniques other than complete excision for treatment of cystic hepatic lesions such as internal drainage, aspiration, marsupialization, sclerosis, Roux-en Y cyst-bowel anastomosis or partial resection should not be performed in cystadenomas because they may result in biliary obstruction, secondary infection or sepsis, rupture, hemorrhage, continued tumor growth, recurrence or late malignant transformation of the tumor^[3,7,10,11,14,17,19,25,45,51]. Benign biliary cystadenomas are believed to transform to cystadenocarcinomas even decades after partial resection although few of these lesions have been reported^[26,36,60]. Moreover, patients with hepatobiliary cystadenomas have been found to be over 10 years younger than those with cystadenocarcinomas^[7]. Cystadenomas should therefore be appreciated as premalignant lesions^[7,10,17,33,45,60] while cystadenoma and cystadenocarcinoma may be considered a different form of the same disease^[15]. Furthermore, cystadenoma cannot be easily differentiated preoperatively or intraoperatively from cystadenocarcinoma and total surgical resection should always be considered^[1,5,6,10,13,33,48,54]. Since complete surgical resection is the only safe way of eliminating such a danger and differentiating the two entities, such tumors should be completely excised^[60,61].

In addition, experience with techniques such as aspiration, fenestration, internal drainage, intratumoral sclerosant application or partial resection of cystadenomas is disappointing since the recurrence rate is extremely high ranging from 90% to 100%^[11,19,21,64,65] compared to 0%-10%^[22,25,26,48,52,60] after radical resection. Similarly, Davies *et al*^[17] reported that the recurrence rate for extrahepatic lesions is 50% after local excision from the bile duct wall compared to no recurrence after formal resection and bilioenteric reconstruction. Moreover, recurrence may be demonstrated even decades after subtotal resection^[51] while consecutive recurrences in the same patient have also been reported^[59].

Though still in evolution, laparoscopic surgery has an expanding role in the treatment of carefully selected patients with liver lesions and can achieve promising results^[66]. There are very few reports on successful laparoscopic treatment of intrahepatic biliary cystadenomas^[14,66] while no case of laparoscopically treated extrahepatic lesion has been reported. Even though laparoscopic treatment of intrahepatic cystadenomas has not been analyzed, provided that it is performed by expert liver as well as laparoscopic surgeons, the patients are carefully selected in terms of tumor size and location, and the tumor is com-

pletely excised, laparoscopic treatment is feasible and safe and may lead to similar results as open surgery^[14,66].

The prognosis of patients with cystadenoma is very good if total excision of the lesion is performed^[5,7,10,25,48,52]. In addition, cystadenocarcinomas may not show aggressive clinical behavior, and usually appear to have a slower growth rate and less frequent metastases or local invasion than other hepatic malignant neoplasms, such as hepatocellular carcinoma and cholangiocarcinoma, and ovarian or pancreatic cystadenocarcinomas^[48]. When treated with radical excision, they also have a generally good prognosis^[48,52], particularly those with mesenchymal stroma, unless the tumor invades the adjacent liver tissue or neighboring organs, or metastases are present^[33,39,48].

CONCLUSION

The natural history of symptomatic progressive tumor enlargement, the inability to preoperatively differentiate cystadenoma from cystadenocarcinoma, the high possibility of recurrence after palliative procedures, and the potential for malignant transformation lend support to the thesis that biliary cystadenoma denotes a potential malignancy. Therefore total resection and a prolonged close follow-up should be performed.

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CASE REPORT

Huge primitive neuroectodermal tumor of the pancreas: Report of a case and review of the literature

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Abstract

Primitive neuroectodermal tumor (PNET) of the pancreas is an extremely rare tumor that usually occurs in children or young adults. We report a case of a 33-year-old male patient with an 18 cm × 18 cm × 16 cm mass arising from the pancreatic body and tail with a one-day history of abdominal pain. Initial CT scan showed no signs of metastatic tumor spread. The tumor caused intrabdominal bleeding and the patient underwent primary tumor resection including partial gastrectomy, left pancreatic resection and splenectomy. Diagnosis of PNET was confirmed by histology, immunohistochemistry and FISH analysis. All neoplastic cells were stained positive for MIC2-protein (CD99). Approximately one month after surgery, several liver metastases were observed and the patient underwent chemotherapy according to the Euro-Ewing protocol. Subsequent relaparotomy excluded any residual hepatic or extrahepatic abdominal metastases. Although PNET in the pancreas is an extremely rare entity, it should be considered in the differential diagnosis of pancreatic masses, especially in young patients. This alarming case particularly illustrates that PNET in the pancreas although in an advanced stage can present with only a short history of mild symptoms.

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Key words: Primitive neuroectodermal tumor; Pancreas; MIC2-protein; Ewing sarcoma; Abdominal mass

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INTRODUCTION

Primitive neuroectodermal tumors (PNETs) are small round cell tumors arising from soft tissue belonging to the Ewing's sarcoma family. These neoplasms all exhibit a neural phenotype, express the MIC2-protein (CD99) and display the same chromosomal translocation t(11; 22)(q24; q12) in about 85% of the cases^[1]. While Ewing sarcoma is a primary bone tumor and follows osteosarcoma as the second most common malignant bone tumor in children, PNETs occur most often in soft tissue of the thoracopulmonary region, pelvis and lower extremities of children and young adults. Most of these tumors are diagnosed before the age of 35 years with a slight predominance in male patients^[1]. Although PNETs can occur in numerous solid organs such as the kidney, ovary, vagina, testis, uterus, cervix uteri, urinary bladder, parotid gland, heart, lung, rectum and pancreas, it is an extremely rare tumor entity^[2]. According to the literature there are currently 11 reported cases of PNET originating from the pancreas (Table 1)^[2-6].

Here we report another case of PNET in the pancreas in an otherwise healthy 33-year old man.

CASE REPORT

A 33-year old man presented to the Emergency Department of our hospital with acute abdominal pain which started one day ago. The patient reported one episode of vomiting but no history of diarrhea, peptic ulcer disease or pancreatitis. Laboratory data on admission showed leucocytosis, elevated C-reactive protein, amylase and lipase.

CT-scan of the abdomen revealed a 13.7 cm × 15.0 cm mass arising from the pancreatic tail compressing the stomach and spleen (Figure 1). The mass had solid and cystic characteristics with an inhomogenous contrast enhancement. Furthermore, blood was noted in the subhepatic area and pelvis indicating rupture of the tumor. Numerous mesenteric lymph nodes were enlarged up to 11 mm, while the liver showed no lesions suspicious of metastasis. Until surgery, a significant decline in hematocrit due to tumor bleeding was observed. At

Table 1 Overview of reported cases of pancreatic PNET

Reference	Age (yr)	Gender	Tumor origin	Tumor size (cm)	Therapy	Survival (cause)
Lüttges <i>et al</i> ^[5] 1997	13	Female	Pancreatic body/tail	22 × 8 × 10	Resection, chemotherapy	NR
Bulchmann <i>et al</i> ^[3] 2000	31	Male	Pancreatic body	-	Chemotherapy, resection	NR
	6	Female	Pancreatic head	4.0 × 5.4 × 3.0	Whipple resection with colon segmentectomy	6 mo (RD)
Movahedi-Lankarani <i>et al</i> ^[2] 2002	6-25 (mean 18)	4 male, 3 female	Pancreatic head	3.5 to 9.0	Whipple resection in 4 cases (2 VDC), biopsy in 3 cases (1 VDC)	Up to 48 mo
Perek <i>et al</i> ^[6] 2003	31	Male	Pancreatic head and body	10 × 12	1 Whipple resection (radiochemotherapy denied), 2 Local resection + chemotherapy, 3 Resection of lung metastasis + chemotherapy	50 mo (RD)
Present case	33	Male	Pancreatic body	18 × 18 × 16	Resection, 6 cycles VIDE, VAI, AST	Alive at 1 year after diagnosis

AST: Autologous stem cell transplantation; mo: Months; NR: Not reported; RD: Recurrent disease; VDC: Vincristin, doxorubicin, cyclophosphamide; VIDE: Vincristin, ifosfamide, doxorubicin and etoposide; VAI: Vincristin, actinomycin D; Ifosfamide.



Figure 1 CT-scan showing a huge intra-abdominal mass with solid and cystic areas originating from the pancreas.

laparotomy the tumor was found to be adherent to the stomach and the pancreatic body and tail. The capsule was partially ruptured. An enbloc-resection of the tumor was accomplished by partial gastric resection (Billroth-II), left pancreatic resection and splenectomy. The continuity was restored with gastrojejunostomy and Roux-Y-jejunostomy. The postoperative course was uneventful.

Grossly, the tumor measuring 18 cm × 18 cm × 16 cm was surrounded by an edematous capsule and appeared as cystic with alternating necrotic areas. The tumor was adhered to the pancreatic tail and stomach adjacent to the minor curvature where two small non-neoplastic ulcers were noted.

On microscopic examination, the solid tumor parts were composed of nests of medium-sized round or oval tumor cells with enlarged round or oval nuclei and scant cytoplasm surrounded by fibrovascular septae (Figure 2A). Focally, Homer-Wright rosettes were observed (Figure 2A). Immunohistochemically, the tumor showed a consistent and strong membranous expression of CD99 (Figure 2B) and a strong cytoplasmic staining for vimentin (Figure 2C). NSE-positive and negative neoplastic cells were found in about equal proportions, and smaller neoplastic

subsets were positive for cytokeratin (KL-1; Figure 2D), cytokeratin 18, EMA, synaptophysin, CD56 (Figure 2E) and CD117. The neoplastic cells lacked any detectable cytokeratins (7, 8 and 19), CEA, α 1-fetoprotein (AFP), α 1-antichymotrypsin (α 1ACT), protein S100, melan A and HMB-45. The Ki67 (MIB-1) labeling index was about 20%-30% (Figure 2F). Fluorescence *in situ*-hybridization was performed with a dual-colour DNA probe flanking the EWS gene on chromosome 22q12 (Vysis/Abbott, Wiesbaden, Germany) according to the manufacturer's instructions. Tumour cell nuclei showed one fused signal and one dislocated hybridization signal indicative of a chromosomal translocation involving the EWS gene. Overall, the microscopic findings led to the diagnosis of PNET.

Following primary hospitalization and surgery the patient was scheduled for staging examinations prior to chemotherapy. Thirty-five days after laparotomy, CT and MRI revealed at least two liver metastases in segments 6 and 7 up to 1.5 cm in size that were biopsied and confirmed immunohistochemically. There were no metastases in the lung, bone marrow or in the skeletal system according to staging by CT, bone marrow biopsy and scintigraphy. The patient underwent 6 cycles of induction VIDE chemotherapy (vincristin, ifosfamide, doxorubicin and etoposide), 1 cycle of VAI chemotherapy (vincristin, actinomycin D, ifosfamide) followed by high dose chemotherapy with melphalan and etoposide and autologous stem cell transplantation. During chemotherapy, devitalization and shrinking of the liver metastases were documented. Because CT visualized a residual lesion in segment 6 of the liver, the patient underwent explorative laparotomy with intraoperative ultrasound which did not show any metastasis or residual tumor. One year after diagnosis, there was no evidence of tumor recurrence.

DISCUSSION

Ewing sarcoma (EWS) and PNET belong to a tumor family (Ewing family of tumors) that is characterized

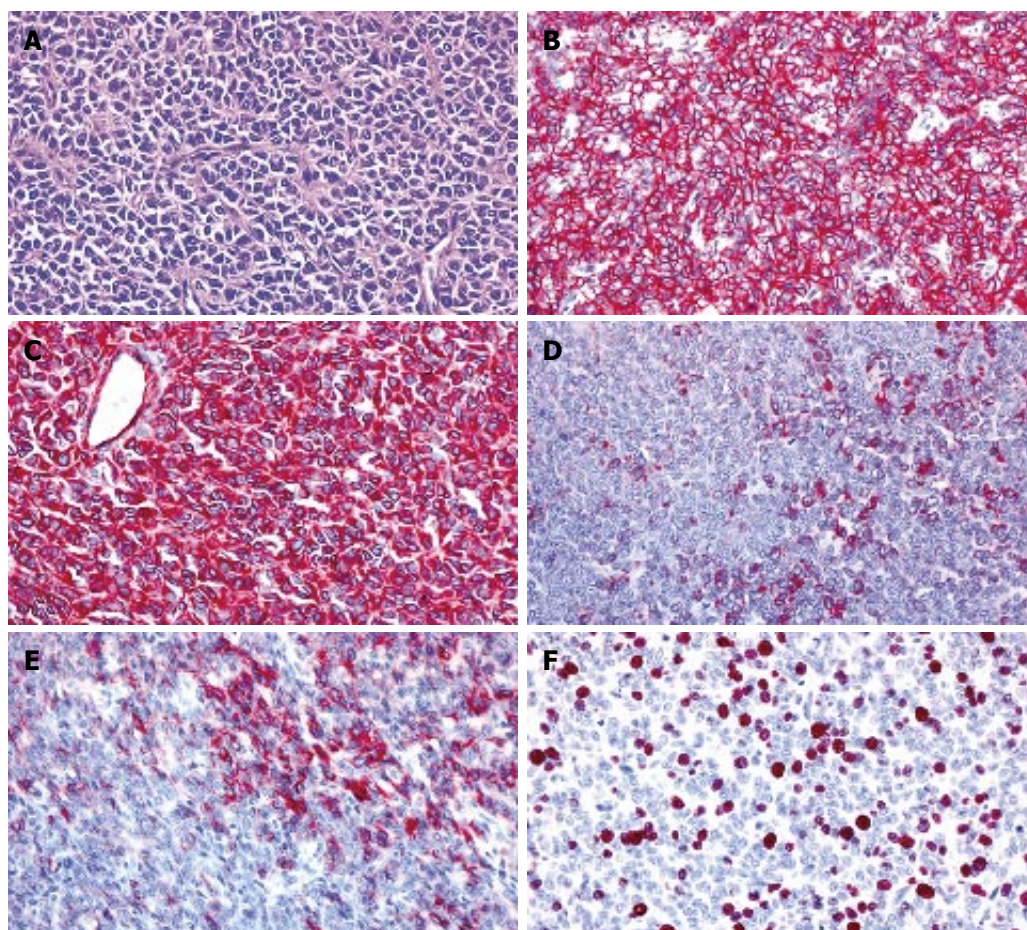


Figure 2 Histomorphological characteristics and immuno-labeling of PNET. **A:** Solid tumor cell sheets separated by a delicate fibrovascular stroma; **B:** Strong membranous expression of CD99 (MIC-2) in the entire tumor cell population; **C:** Vimentin-positive neoplastic cells; **D:** Expression of cytokeratins (KL-1) in a minor tumor cell subset; **E:** Partial CD56-positive neoplastic population; **F:** Nuclear staining for the Ki67 proliferation antigen (MIB-1) in about 20%-30% of tumor cells.

by typical chromosomal translocations with subsequent functional fusion of the EWS gene with transcription factor genes thus forming a chimeric protein. Peripheral PNET (pPNET) was first described by Stout^[7] as a tumor of the ulnar nerve with the gross features of a sarcoma but composed of small round cells focally arranged as rosettes in 1918. pPNET makes up approximately 1% of all sarcomas^[2]. EWS and PNET form a subset of the “small-round-cell tumors” in childhood that comprise lymphomas, neuroblastoma and soft tissue sarcomas including rhabdomyosarcomas as well, which at least in undifferentiated forms, are indistinguishable by conventional light microscopy. When PNET is found in the pancreas, the differential diagnosis includes undifferentiated small cell carcinoma, pancreatoblastoma and pancreatic endocrine tumors. Thus the diagnosis of PNET necessitates histopathologic, immunohistochemical and if possible, also genetic analysis. EWS and PNET show a high expression of the cell surface glycoprotein MIC2 (also named CD99 or p30/32^{MIC2}) which is considered to be important in cell adhesion^[1]. However, other neoplasms as well as normal tissue including pancreatic endocrine tumors and pancreatic islet cells express the MIC2 protein, thus limiting specificity of the test.

Cytogenetic and molecular analysis of translocations has been established as a powerful adjunct for sarcoma classification. EWS/PNET show typical chromosomal translocations involving the EWS gene on chromosome 22

and a member of the ETS family of genes which code for DNA-binding transcription factors. The most common translocation $t(11; 22)(q24; q12)$ resulting in the fusion product EWS-FLI1 occurs in 85%-95%, while the second most common translocation $t(21; 22)(q22; q12)$ is seen in 5%-10%^[1].

Once PNET is diagnosed, the standard treatment is a systemic multi-agent chemotherapy combined with surgery and/or radiotherapy. Tumor dissemination at the time of diagnosis is associated with a poorer outcome compared to localized disease^[8]. However, a retrospective study on 24 patients younger than 16 years old with extraskelatal EWS reported that age and surgical treatment, but not size of tumor and metastatic disease at the time of diagnosis are significant prognostic factors^[9]. In this study the overall 5-year survival rate was 61% and the disease free survival rate was 54%. In various series including patients with extraskelatal EWS, the 5-year survival rate ranges from 61% to 77%^[8]. Intensive preoperative multi-agent chemotherapy may further improve the prognosis of EWS family tumors^[10]. There are only few data focusing on the prognosis of patients with PNET in the pancreas. Movahedi-Lankarani *et al*^[2] reported 2 patients with chemotherapy after primary Whipple procedure. One was alive at 33 mo with no evidence of disease, while the other died of disease 4 years after diagnosis.

In conclusion, PNET of the pancreas is an extremely rare pancreatic tumor and mainly affects children or young adults. To our knowledge, this is the 12th-reported case so

far. Although uncommon, PNET has to be considered in the differential diagnosis of atypical pancreatic tumors in young patients.

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CASE REPORT

Intracolonic multiple pebbles in young adults: Radiographic imaging and conventional approach to a case

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Abstract

Most of the foreign bodies detected in adult gastrointestinal systems are accidentally swallowed pins. In this study, we presented a case with intracolonic multiple pebbles. A 20-year-old man was admitted to emergency surgery policlinic for abdominal pain for 2 d without any alleviation or aggravation. His upright plain abdominal radiographic imaging revealed about 30-40 overt dense opacities in lumen of colonic segments, with oval and well shaped contours, each approximately 1 cm x 1 cm in size. The multiplanar reconstructions and three-dimensional images combined with sectional screening showed that all pebbles had passed completely into the colon and no foreign bodies had remained in the ileal segments. On psychiatric assessment, he was found to have immature personality features, difficulty in overcoming stressors and adaptation disorder. He recovered by conservative management and radiographic monitoring applied during his follow-up. Thus, it can be concluded that, in differential diagnosis of abdominal pain in adult ages, though less frequently seen than in children, gastrointestinal system foreign bodies should always be kept in mind and it should be considered that ingestion of pebbles may be one of the factors contributing to abdominal pain particularly in young adults with psychiatric problems. In such cases suspected of having foreign bodies which cannot be detected by plain films, abdominal tomography can be an alternative for diagnostic imaging.

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Key words: Intracolonic multiple pebbles; Current approach; Radiographic imaging

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INTRODUCTION

The frequency of foreign body detection in gastrointestinal system is less in adulthood compared to childhood. Accidentally swallowed pins account for most of the foreign bodies found in adult gastrointestinal systems. Oral dental implants, bezoars, chicken-fish bones, packages of medications and drugs are among the other foreign bodies commonly seen to be swallowed^[1]. Incidence of foreign bodies swallowed by an individual with mental defect or due to conversive reaction is relatively rarer. Regardless of the types of the objects, foreign bodies are very rare to result in a serious clinical condition in intestinal lumen. If it does not develop an emergency surgical indication, there is no need to hospitalize the cases. Conservative follow-up approach is a generally preferred method. Out-patient follow-up through serial physical examination and plain films will be enough. A good observation and quick radiographic monitoring is suggested for the follow-up of such patients. As long as the objects do not get stuck in intestinal transition points such as pylorus or ileo-caecal valve, they are generally passed rectally out within a few days^[2]. We present herein a young adult case of intracolonic multiple pebbles, who was managed successfully by serial radiographic imaging and conventional approach, without surgical intervention.

CASE REPORT

A 20-year-old male presented in a peripheral hospital with the complaint of abdominal pain. With a provisional diagnosis of "intestinal foreign body", based on his upright abdominal plain film, he was referred to our hospital. The abdominal pain had been continuing for 2 d, with mild severity without any alleviation or aggravation. His physical examination revealed soft abdomen, no guarding and rebound tenderness, and normal bowel sound. However, tenderness was found at right lower quadrant on deep palpation. No abnormality was detected in rectal examination. Complete blood count and urine test were within normal limits. His upright abdominal plain film showed about 30-40 overt dense opacities in the colonic segments, with oval and well shaped contours, each approximately 1 cm x 1 cm in size (Figure 1). In addition to these multi-opacities, an image of a pin was detected in the region consistent



Figure 1 An upright plain abdominal radiographic imaging revealing 30-40 overt dense opacities in lumen of colonic segments, with oval and well shaped contours, each approximately 1 cm x 1 cm in size.



Figure 2 CT showing dense opacities in lumen of colonic segments, with oval and well shaped contours.



Figure 3 A multiplanar reconstruction and three-dimensional image combined with sectional screening showing all pebbles had passed completely into the colon and no foreign bodies were remained in the ileal segments.

with the right lower quadrant. In abdominal ultrasound, there were not any obvious abnormalities supporting the findings of the plain film. However, a minimal amount of smear-like fluid was present around the ileal segments in right lower quadrant. Low-dose computed abdominal tomography was applied without administering contrasting substance to see whether there is a foreign body which is unable to pass into the colon and poses the risk of obstruction, to be able to detect other foreign bodies which might not show any opacity, to evaluate the ileo-caecal valve better, and to better analyze the smear-like fluid shown by ultrasonography. In the lumen of colon, about 30-40 hyperdense images, suspicious of being pebbles, were detected (Figure 2). The multiplanar reconstructions and three-dimensional images combined with sectional screening revealed that all pebbles had passed completely into the colon and no foreign bodies had remained in the ileal segments (Figure 3). After computed abdominal tomography, rectal enema was applied to the patient and a few pebbles were passed out the body through defecation. He was managed conservatively and monitored with physical examination and abdominal plain film on follow-up in surgical out-patient clinic, until all the pebbles and pin were discharged within one week *via* defecation without any difficulty.

DISCUSSION

Intestinal foreign body cases resulting from foreign body ingestion accidentally or due to a compulsive reaction are seen less frequently in adulthood when compared to childhood. Types of the intestinal foreign bodies taken orally

by adults differ from those taken by children. In literature, plastic pipe, metal pin, nail, screw, spoon, bamboo stick and paper clip have been reported in adults as gastrointestinal system foreign bodies^[3-5]. Some case reports are available regarding Meckel's diverticulum perforations caused by swallowed fish bone^[6-8], foreign bodies^[9-11], chicken bone^[12] and alkaline battery^[13]. In the relevant literature, Selivanov *et al.*^[14] published a series of 101 and Steven *et al.*^[15] a series of 75 foreign body ingestion cases. In addition, Losanoff *et al.*^[5] presented a series of 9 prisoners who swallowed metal parts shaped like a cross.

Orally ingested foreign bodies are the cases that are not seen frequently in emergency surgery polyclinic. The basic approach is physical examination and imaging techniques. Patients considered not having acute abdominal syndrome after physical examinations are followed up with a serial upright abdominal plain film and physical examination. In case the foreign body cannot be visualized in conventional films, it should be kept in mind that the patient did not swallow the object or conventional techniques remained inadequate to visualize it. When the presence of other foreign bodies is suspected although one is observed on plain films, computed abdominal tomography can be considered as a proper alternative^[16-20]. Plain film findings of our case were evaluated in accordance with literature. Abdominal ultrasonography was applied considering that there might be foreign bodies which cannot be seen on plain films. Pebbles and the pin detected on plain films could not be seen on ultrasonography. Then computed abdominal tomography was applied to the case, which revealed multiple opacities that were suspected of being pebbles with intracolonic localization.

Intestinal foreign body cases, regardless of being in adulthood or childhood, do not reveal any symptoms as long as the object does not cause obstruction. If they lead to any obstruction or perforation, the main symptom to emerge will be abdominal pain, as was found in our case. Data have shown that majority of the cases are normal adults who swallowed fish or chicken bones while eating or mentally retarded patients who swallowed foreign bodies unconsciously.

His physical examination revealed soft abdomen, no guarding and rebound tenderness, and normal bowel sound. However, tenderness was found at right lower quadrant on deep palpation. No acute abdominal symptom or a clinical condition requiring an emergency surgi-

cal intervention could not be obtained in the case. The swallowed foreign bodies were clearly seen on his upright abdominal plain film. Literature points out that metal and glass objects can be seen on plain films, but not wooden objects, so abdominal USG may be obtained when deemed necessary^[21]. It will be very helpful to evaluate the abdomen through computed tomography in addition to plain films particularly in cases with psychiatric problems, suspicious of swallowing objects such as drugs, posing the risk of harming themselves and in those trying to conceal the truth, as seen in our case. Computed tomography and three-dimensional imaging were applied to the case in order to reveal other foreign bodies that might be present although they could not be monitored on plain films and to eliminate the foreign bodies that might remain in ileal segments and lead to ileo-caecal obstruction. Since there might be some other foreign bodies that cannot be detected by plain films, computed tomography and three-dimensional imaging are applied as the appropriate alternative for the detailed assessment of the intestinal lumen in suspected problematic cases and in the elimination of probable future complications.

While approaching to the intestinal foreign body cases, location of the foreign body has a great importance in terms of management. According to the localization and shape of the object, it is decided on whether an out-patient treatment or surgical intervention will be applied^[17,20,21]. Although sharp objects pass through gastrointestinal tract without difficulty in 90% of the cases, they may also be removed using fiberoptic gastroscopy without waiting. Early endoscopic intervention, in general, may be required and applied for objects wider than 2 cm and longer than 6 cm, since their passage through pylorus and duodenum will become difficult. Once these foreign bodies pass beyond pylorus and ileo-caecal valve, they are easily removed out of the lumen^[2]. Losanof *et al*^[12] operated all cases since the foreign bodies have pointed and sharp edges. Our case was managed conservatively and monitored on out-patient polyclinic follow-up, in the light of the literature. He was informed about the probable progress of his condition and suggested to be admitted to the clinic as soon as possible in case of acute abdominal pain and/or any other complaints.

In conclusion, in differential diagnosis of abdominal pain seen in adult ages, gastrointestinal system foreign bodies should always be kept in mind although they are seen less frequently than in children. It should be considered that intestinal system foreign bodies may be one of the factors contributing to abdominal pain particularly in young adults. In such cases suspected of having foreign bodies which cannot be detected by plain films, abdominal

tomography can be a sound alternative for diagnostic imaging.

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Sigmoid colonic carcinoma associated with deposited ova of *Schistosoma japonicum*: A case report

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Abstract

We report a case of sigmoid colonic carcinoma associated with deposited ova of *Schistosoma japonicum*. A 57-year old woman presented with a 10-mo history of left lower quadrant abdominal pain and a 2-mo history of bloody stools. She had a significant past medical history of asymptomatic schistosomiasis japonica and constipation. A colonoscopy showed an exophytic fragile neoplasm with an ulcerating surface in the sigmoid colon. During the radical operative procedure, we noted the partially encircling tumor was located in the distal sigmoid colon, and extended into the serosa. Succedent pathological analysis demonstrated the diagnosis of sigmoid colonic ulcerative tubular adenocarcinoma, and showed deposited ova of *Schistosoma japonicum* in both tumor lesions and mesenteric lymph nodes. Three days after surgery the patient returned to the normal bowel function with one defecation per day. These findings reveal that deposited schistosome ova play a possible role in the carcinogenesis of colorectal cancer.

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Key words: Schistosomiasis japonica; Sigmoid colonic carcinoma; Deposited schistosome ova

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INTRODUCTION

Schistosomiasis is a trematode parasitic infection in which terminal hosts are humans or other mammals with freshwater snails as intermediate hosts^[1]. Schistosomiasis

japonica is endemic mainly in China, and remains a major public health problem today although remarkable successes in schistosomiasis control have been achieved over the previous four decades^[2].

The life cycle of *Schistosoma japonicum* (*S. japonicum*) starts when cercariae in the freshwater infect humans or other mammals. After maturing, male and female worms pair up in the hepatic portal vein system, and produce ova in the mucosal branches of the inferior mesenteric and superior hemorrhoidal veins. Many ova are discharged in the feces, hatch, and release free-swimming miracidia, which in turn reinfect receptive freshwater snails and asexually produce larval cercariae^[2]. However, some ova deposit in the tissues of the host, cause host inflammatory responses, and lead to granuloma formation which is the principal pathology associated with schistosomiasis^[3]. Periovarian granulomas have been found in many types of tissue, including the liver, intestine, skin, lung, brain, adrenal glands, and skeletal muscle. Ova retained in the gut wall induce inflammation, hyperplasia, ulceration, microabscess formation, polyposis, and carcinogenesis^[3].

Successes in schistosomiasis control in China have prolonged the life of patients with schistosomiasis japonica in which the chronic pathological process may occur due to deposited schistosome ova.

Here we report a case of sigmoid colonic carcinoma associated with deposited ova of *S. japonicum*, with their possible role in carcinogenesis discussed.

CASE REPORT

A 57-year old woman presented with a 10-mo history of left lower quadrant abdominal pain and a 2-mo history of bloody stools. She had a sense of pain in the left lower quadrant abdomen when she had bowel movements about 10 mo ago. The pain was relieved after defecation, so that she did not think much of it. Two months ago, she noted that dark red blood adhered to the surface of stools without blood drops from anus. The symptom of hematochezia persisted until hospitalization. She had no other complaints. She had a significant past medical history of asymptomatic schistosomiasis japonica and constipation. She came from the rural area in Zhejiang Province, where schistosomiasis japonica was prevalent several decades ago. She had the experience of swimming in a local river in her childhood. At the age of 13, she was diagnosed with schistosomiasis through a thick smear stool examination by the county public health workers

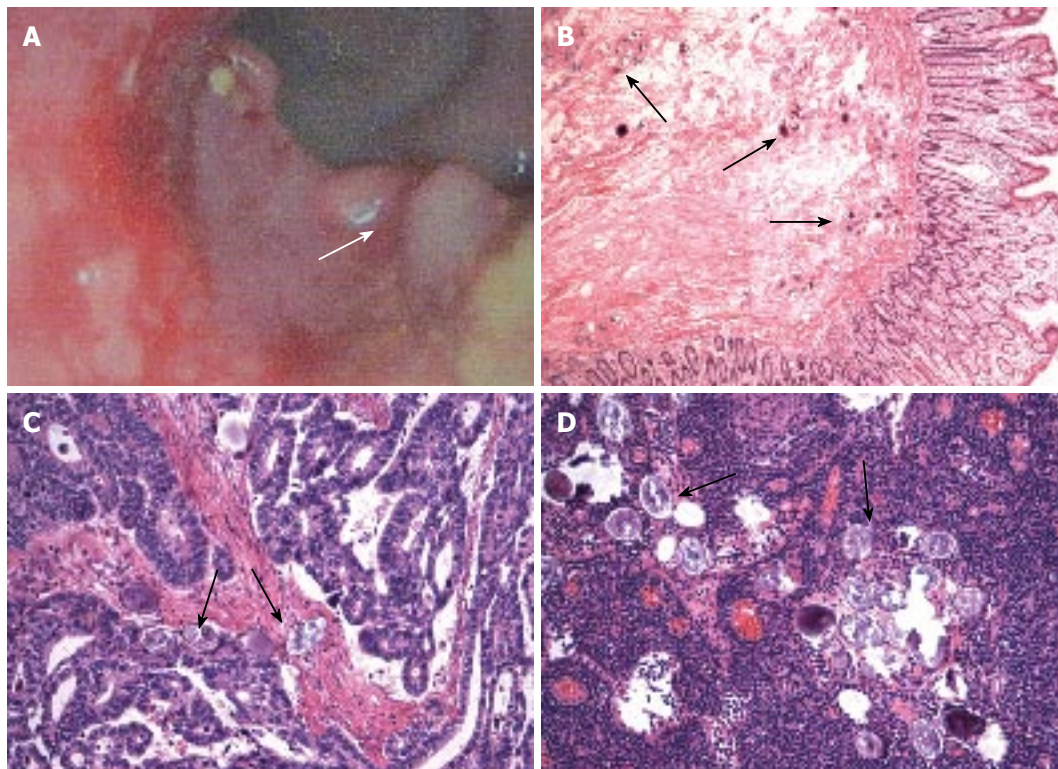


Figure 1 Colonoscopy showing an exophytic fragile neoplasm with an ulcerating surface (as indicated by the white arrow head) in the sigmoid colon (A), pathological analysis revealing deposited *S. japonicum* ova and granuloma formation as well as fibrotic deposition in the submucosa of the sigmoid colon (B) (HE \times 50), moderately-differentiated tubular adenocarcinoma (HE \times 200) and deposited *S. japonicum* ova in the tumor (C), and deposited *S. japonicum* ova in the mesenteric lymph node (D) (HE \times 200). Black arrow heads indicate *S. japonicum* ova.

for schistosomiasis control, and given the appropriate antischistosomiasis therapy which was not terminated until repeated stool examinations were negative. At the age of 20, she suffered from constipation which was not improved by adequate fiber intake. Usually, she had about two bowel movements with hard stools per week. The symptom began to become worse ten years ago and she had defecation once per week.

She was in good general condition. No supraclavicular lymph nodes were palpable. On abdominal palpation, there was a discomfortable sense in the left lower quadrant without palpable masses. Her peripheral blood cell count showed leucopenia (white blood cells: $3100/\text{mm}^3$) and mild thrombocytopenia (platelets: $72000/\text{mm}^3$), without anemia and eosinophilia. Three stool tests were all negative for *S. japonicum* ova. Liver function, renal function and electrolyte concentration were all normal. Tumor markers (AFP, CEA, and CA-199) were 2.41 ng/mL, 0.72 ng/mL, and 5.88 U/mL, respectively, which were within normal limits. The abdominal ultrasonography revealed echodense areas scattered within the parenchyma with typical fish-scale pattern, representing the trait of liver schistosomiasis. The computed tomographic scan showed that the bowel wall of sigmoid colon was incrassated and irregular in shape. The colonoscopy showed an exophytic fragile neoplasm with an ulcerating surface in the sigmoid colon (Figure 1A), which was diagnosed as adenocarcinoma through biopsy.

After definite diagnosis was made, the radical operative procedure was performed. During laparotomy, the entire peritoneal cavity was examined, with a thorough inspection of the liver, pelvis, and hemidiaphragm. Neither metastatic lesions nor enlarged mesenteric lymph nodes were found. The partially encircling tumor was located in the distal

sigmoid colon, and extended into the serosa. Pathological analysis of the operative specimen revealed deposited *S. japonicum* ova, granuloma formation, and fibrotic deposition in the submucosa of the sigmoid colon (Figure 1B), and demonstrated a grade 2 moderately-differentiated sigmoid colonic ulcerative tubular adenocarcinoma, 3 cm \times 2 cm in size, penetrating the serosa without lymph node involvement (Figure 1C). Deposited *S. japonicum* ova were found in both tumor lesions (Figure 1C) and mesenteric lymph nodes (Figure 1D).

Three days after surgery the patient returned to the normal bowel function with one defecation per day.

DISCUSSION

The relation between colorectal cancer and schistosomiasis japonica has not been well established. However, researchers in China have noted the coincidence of colon cancer and schistosomiasis japonica at the patient level during the 1970s^[4]. Another case-control study in Jiangsu Province showed that rectal cancer is associated with schistosomiasis japonica^[5]. A recent matched case-control study showed that previous *S. japonicum* infection is significantly associated with both liver cancer and colon cancer^[6]. It was also reported that schistosomiasis japonica correlates with colorectal cancer^[7].

The mechanism of schistosome infection-induced colorectal cancer was explored in our study. The mutations in p53 tumor suppressor gene in Chinese patients with schistosomiasis japonica-related rectal cancer and a high proportion of base-pair substitutions at CpG dinucleotides and a higher frequency of arginine missense mutations were observed, suggesting that the mutations are the result of genotoxic agents produced endogenously

through the course of schistosomiasis japonica^[8]. Another study showed that *S. japonicum* ova-induced colorectal epithelial proliferative polyps have a high percentage of atypical hyperplasia (64.9%) and CEA (90%)^[9]. Thus the epithelial proliferative polyp, especially that with atypical hyperplasia, is the transition during deposited ova-induced carcinogenesis. It was reported that the majority of colorectal cancer cases associated with *Schistosoma mansoni* (*S. mansoni*) infection have a significant expression of Bcl-2 while p53 and C-Myc expressions are insignificantly different in colorectal cancer cases associated with *S. mansoni* infection compared with those without *S. mansoni* infection^[10]. In a word, the mechanism of schistosome infection-induced carcinogenesis remains unclear.

In our present case, both chronic constipation and sigmoid colonic adenocarcinoma were induced by deposited *S. japonicum* ova, revealing that ova can interact with the host microenvironment. Asymptomatic schistosomiasis in our patient was cured as neither eosinophilia nor ova in her stools were observed. However, many old ova deposited in sigmoid colonic submucosa, tumor lesions, and mesenteric lymph nodes. Furthermore, the anatomic location of adenocarcinoma corresponded to the colorectal area with most deposited ova. These findings indicate that deposited *S. japonicum* ova play a possible role in the carcinogenesis of colorectal cancer^[7].

Another important aspect of this case is that chronic constipation was improved after operation. In this case, granuloma formation and fibrotic deposition around deposited ova perhaps disturbed the sensory function of the sigmoid colon, and resulted in constipation.

Additionally, we noted deposited ova in the mesenteric lymph nodes, leucopenia, and thrombocytopenia in this case. However, their pathological significance remains unclear.

In conclusion, sigmoid colonic carcinoma is associated with deposited ova of *S. japonicum*. Therefore, screening

for colorectal cancer should always be thoroughly performed with routine endoscopy in patients with previous *S. japonicum* infection, and the mechanism of deposited *S. japonicum* ova-induced carcinogenesis should be further investigated.

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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
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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

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Society for Diseases of the Esophagus
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isde@sapmea.asn.au
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- 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]

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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 Xiaohua Zazhi* 1999; **7**: 285-287

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- 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

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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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- 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

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- 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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Gene therapy of liver cancer

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Abstract

The application of gene transfer technologies to the treatment of cancer has led to the development of new experimental approaches like gene directed enzyme/pro-drug therapy (GDEPT), inhibition of oncogenes and restoration of tumor-suppressor genes. In addition, gene therapy has a big impact on other fields like cancer immunotherapy, anti-angiogenic therapy and virotherapy. These strategies are being evaluated for the treatment of primary and metastatic liver cancer and some of them have reached clinical phases. We present a review on the basis and the actual status of gene therapy approaches applied to liver cancer.

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Key words: Gene therapy; Cancer; Liver; Hepatocellular carcinoma; Vector; Therapeutic gene

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INTRODUCTION

Knowledge of molecular mechanisms governing malignant transformation brings new opportunities for therapeutic intervention against cancer using novel approaches. One of them is gene therapy. This new discipline is based on the transfer of genetic material to an organism with the aim of correcting a disease. The genes can be delivered directly into the subject, using a variety of vehicles named vectors (*in vivo* gene therapy), or delivered into isolated cells

in vitro that are subsequently introduced into the organism (*ex vivo* gene therapy).

Cancer is the most frequent application of experimental gene therapy approaches^[1] for several reasons.

First, the genetic alterations that give rise or contribute to the malignant transformation of cells are being unravelled with increasing detail in the last two decades, and this provides multiple candidate targets for gene therapy intervention^[2]. Nevertheless, the genetic and epigenetic alterations that lead to an established tumor are complex and require special approaches that often differ from gene therapy applied for hereditary monogenic diseases. In many cases, the transfer of genes into malignant cells is not performed with the intention of correcting a genetic deficiency related to cancer. To be efficient, this would require that the selected gene plays a dominant role in the malignant phenotype. In addition, a technique would be needed that achieves successful modification of virtually every cell in the tumor, something that is far from being realistic in the near future. Therefore, different strategies have been developed to introduce genes that cause the destruction of the tumor by indirect mechanisms, as will be discussed below.

Second, some tools initially developed as gene therapy vectors, such as certain viruses, are being exploited as oncolytic agents by themselves. This means that the development of gene therapy approaches against cancer is activating other fields such as virotherapy and cellular therapies.

Finally, the lack of safe and efficient therapeutic options against many types of cancer is fostering the development of new gene therapy applications for these diseases. Liver cancer is a good example of this situation. Hepatocellular carcinoma (HCC) accounts for 80% of primary liver tumors in adults, has an increasing incidence^[3] and a poor 5-year survival rate of about 7% despite treatment^[4]. In addition, the liver is the most frequent site of metastasis, especially from gastrointestinal cancer. Potentially curative therapies such as liver transplantation and surgical resection can only be applied to a minority of subjects because of advanced disease at the time of diagnosis and the lack of suitable organ donors. Other regional treatments may be beneficial for unresectable HCC, but recurrence is frequent and the long term survival rate remains poor. These treatments include transarterial embolization (TACE), percutaneous Ethanol injection, radiofrequency thermal ablation, microwave coagulation therapy, laser-induced thermotherapy and radiotherapy. New protocols based on combinations of regional treatments (with or without previous surgery) are being investigated for the management of HCC, and they are showing a clear advantage when compared to single treatments^[5]. In this context,

gene therapy could be considered as a potential adjuvant to other therapies. The clinical trials performed so far have shown that the side effects are acceptable in most of the cases, and that the mechanism of action is different from standard treatments^[6]. Therefore, choosing the right combination among gene therapy approaches and conventional treatments may achieve a synergistic effect. Furthermore, the refinement of interventional therapies for HCC such as TACE and PEI provides new possibilities for the delivery of gene therapy vectors into hepatic tumors, increasing the effective dose and minimizing potential side effects derived from non-target cell transduction.

In this review we present an insight into gene therapy strategies against liver cancer and discuss the latest developments in the field.

RESTORATION OF TUMOR SUPPRESSOR GENES

This strategy is the most intuitive application of gene therapy for the treatment of HCC and other cancers. It is clear that the loss of function of certain genes (caused by deletions, mutations, promoter inactivation or other epigenetic changes) is associated with malignant transformation of cells^[7]. These tumor suppressor genes control cell proliferation and apoptosis in order to maintain an equilibrated turnover of cells in each tissue. Under experimental conditions (mostly *in vitro*), it has been demonstrated that the restoration of tumor suppressor genes can revert the malignant phenotype of cells^[8]. However, the therapeutic application of this observation faces enormous difficulties. Cancer cells often suffer some degree of genetic instability. When they lose their capacity to sense and repair damaged genes, mutations accumulate and cells with higher proliferation rate and lower sensitivity to apoptotic stimuli are selected sequentially. Under these circumstances, they may become insensitive to the restoration of a particular tumor suppressor gene. On the other hand, this approach requires the introduction of the gene and the expression of the antitumoral protein in virtually all cancer cells, or at least in those responsible for tumor maintenance. This is technically impossible with current gene therapy vectors, especially for solid tumors like HCC.

Despite all these considerations, the transfer of p53 tumor suppressor gene has shown effect in several animal models of cancer, including HCC^[9,10]. This proof of concept has stimulated the use of p53 as a therapeutic gene. Mutations in p53 or alterations in its pathway have been described in more than 50% of human cancers^[11,12]. When cells lack functional p53, they are unable to stop the cell cycle or trigger apoptosis in response to DNA damage. They accumulate mutations that lead to malignant initiation, progression and resistance to treatments. Thus, the restoration of p53 may render tumor cells sensitive to apoptotic stimuli, even if they have accumulated other mutations. This may explain the therapeutic effect observed in pre-clinical models, and suggests a potential role of p53 as an adjuvant to conventional therapies that induce apoptosis in cancer cells.

In contrast, several clinical trials based on delivery of the wild type p53 gene using different vectors have observed variable, and often less positive results in different types of cancer such as lung, head and neck, bladder, ovarian and breast cancer^[13]. However, a first-generation adenoviral vector expressing the p53 cDNA under the control of the CMV promoter became the world's first commercially licensed gene therapy product (Gendicine) for the treatment of head and neck squamous cell carcinoma in China^[14]. In a clinical trial performed on 30 HCC patients, Partial Response (PR) was reported in 2 cases, Stabilized disease (SD) in 24 patients and Progressive Disease (PD) in 4 of them. The virus was administered at doses of $1-2 \times 10^{12}$ VP/wk for 4 wk. In another HCC clinical trial, Gendicine was used in combination with TACE (5FU, HCPT and ADM). The viral doses were similar ($1-4 \times 10^{12}$ VP/wk for 4 wk), starting 2-5 d after TACE. The authors reported 67.6% PR in the combination group versus 51.2% in the TACE-only group (reviewed by Peng^[14]). The clinical significance of these results is controversial at this time, but the availability of a gene-based therapy in the market with potential effect on HCC will probably extend its use in combination with other therapies and allow the identification of synergistic effects. Optimization of the vectors and the therapeutic regimes may be needed to increase gene transfer. For instance, weekly injections of a first-generation adenoviral vector are most likely eliciting a strong immune response that blocks the infectivity of repeated doses after 2 wk. A better understanding of genetic alterations in each particular case of cancer will help to predict the response to the restoration therapy and aid in the selection of candidate patients.

In addition, new strategies are being developed to address the limitations of this approach. One of them relies on the fusion of p53 with VP22, a tegument protein from Herpes Simplex Virus-1 (HSV-1)^[15]. VP22 is exported from the cytoplasm of the expressing cell and gets incorporated by neighbouring cells by poorly defined mechanisms. The fusion of VP22 with other polypeptides enables the intercellular spread of the chimeric protein. It has been demonstrated that an adenoviral vector expressing the p53-VP22 fusion construct achieves higher transduction efficiency and therapeutic effect on a rat model of HCC when compared with wild type p53^[16]. In addition, the combination of p53 with other tumor suppressor genes like p16 can cooperate to induce apoptosis in cancer cells^[17].

The suppressor of cytokine signalling 1 (SOCS-1) gene has been recently identified as a potential tumor suppressor for HCC. Its promoter is frequently inhibited by methylation in HCC, causing activation of the JAK/STAT pathway^[18]. At least *in vitro*, the restoration of SOCS-1 function induces apoptosis in cancer cells.

INHIBITION OF ONCOGENES

The rationale of this approach is in line with the previous one. In this case the correction of the imbalance between positive and negative proliferation signals is attempted by inhibiting the function of genes implicated in the maintenance of cell growth.

nance of unrestricted cell proliferation and acquisition of metastatic phenotype. Many of the drawbacks mentioned above can be applied here, like the need for a highly efficient gene transfer method and a dominant role of the target gene in malignant transformation. The number of candidate oncogenes is continuously expanding as the knowledge of cancer at the genomic and proteomic levels advances^[2].

Hopefully, the inhibition of oncogene expression will not only decrease cell proliferation, but also restore sensitivity of cells to apoptotic stimuli. For instance, it is known that the inhibition of the Ras oncogene, apart from blocking a cascade of mitotic signals, relieves the repression exerted on the p53 pathway and predisposes cells to apoptosis^[19]. This may be the case for other oncogenes such as the pituitary tumor transforming gene 1 (PTTG1)^[20].

Another example is the catalytic subunit of Telomerase (Telomerase Reverse Transcriptase, TERT). Since telomerase function is necessary to maintain telomere length in each cell division, cancer cells undergoing unrestricted cell proliferation present activation of TERT expression^[21]. Therefore, inhibition of TERT is hypothesised to cause inhibition of cell growth after several divisions, when telomeric repeats finally run out. However, efficient inhibition of telomerase expression is able to induce apoptosis in a few days, and this is irrespective of its telomere-lengthening function^[22]. New data indicate that telomerase is also necessary to protect chromosome ends from being recognized as DNA disruptions, which would trigger apoptosis^[23].

Different methods are used to inhibit expression of oncogenes. One of them is based on the transfer of antisense nucleotides, artificial sequences complementary to the mRNA corresponding to the gene whose inhibition is attempted^[24]. These can be short sequences (antisense oligonucleotides, ASO), or the full cDNA. Several mechanisms account for the blocking of gene expression, with the most widely spread and studied being the degradation of RNA-DNA hybrids by cell nucleases such as RNase H. A more recent approach is RNA interference, another posttranscriptional gene silencing mechanism based on the production of double-stranded stretches of RNA complementary to the target mRNA^[25]. Using the endogenous cell machinery, the double-stranded RNA is processed into 21 to 23-nucleotide short interfering RNAs (siRNAs) that recognize the cognate mRNA and trigger its degradation. Alternatively, the siRNAs can be transfected directly. In the "triple helix" strategy, the inhibitory oligonucleotides (triplex-forming oligonucleotides, TFOs) are targeted to the cellular double-stranded DNA^[26]. They interact with polypurine-polypyrimidine sequences in the minor or major groove of genomic DNA and block gene expression at different levels depending on the localization of the complementary sequence. They could be potentially used not only for gene expression modification, but also in gene correction strategies^[27]. Finally, the expression of secreted or intracellular antibody-based molecules has been proposed to block the function of oncogenes^[28,29].

In the case of HCC, the inhibition of several genes has shown potential antitumor effect. Most reports provide

proof of concept showing growth inhibition or induction of apoptosis using HCC-derived cell lines in cell culture. The *in vivo* studies performed in animal models show growth retardation in tumors, especially when cancer cells are transfected *ex vivo*, but complete eradication is difficult when *in vivo* gene therapy is tested on pre-existing tumors.

Since telomerase and Wnt pathway activation are frequently associated with HCC, different approaches including antisense molecules and siRNA have been used to inhibit them^[30-32]. Antisense technology was also used against FGF-2^[33], VEGF^[34] and COX-2 genes^[35]. The triplex helix approach showed similar results as antisense technology for the inhibition of IGF-I and induction of apoptosis in HCC cells^[27]. When the cells were injected into mice, an immune-mediated antitumor protection was observed. The inhibition of PTTG1 and urokinase-type plasminogen activator (u-PA) has been accomplished using siRNA on HCC cells^[20,36]. The p28-GANK oncoprotein, which induces hyperphosphorylation and increases degradation of pRB was found to be overexpressed in the majority of HCCs^[37]. The repeated administration of an adenoviral vector that induces the production of siRNA against p28-GANK caused a dramatic decrease in the growth of human HCC xenografts in nude mice^[38]. This shows that the continuous inhibition of an oncogene may have a strong impact on the progression of tumors. The clinical application of this approach is challenging, because highly efficient long-term expression vectors will be needed instead of first generation adenoviruses.

GENE-DIRECTED ENZYME/PRO-DRUG THERAPY (GDEPT)

This approach is based on the transfer of exogenous genes that convert a non-toxic pro-drug into a cytotoxic metabolite in cancer cells^[39]. Once the pro-drug is administered systemically, transduced cells expressing the converting enzyme die and, in some cases, provoke the destruction of surrounding cells (bystander effect). Unlike other gene therapy strategies, GDEPT lacks intrinsic tumor specificity, and relies on tumor targeting at the levels of cell transfer (depending on the vectors and the route of administration) and gene expression (depending on tumor-specific promoters)^[40]. The efficacy of a GDEPT system is highly influenced by the extent of the bystander effect, because the fraction of transduced cells in a tumor is generally low with current gene therapy vectors^[41].

The thymidine kinase gene from HSV-1 (HSV-TK) used in conjunction with the pro-drug ganciclovir (GCV) was the earliest and most used GDEPT system applied to HCC and other cancers^[42]. It has shown significant antitumor effect in relevant animal models of HCC, such as carcinogen-induced HCC in rats^[43]. HSV-TK converts ganciclovir into the monophosphate intermediate that is subsequently transformed into the triphosphate form by cellular enzymes. This is a highly polar molecule that cannot diffuse outside the cell. The bystander effect of this system has been explained by gap junction transfer of the toxic metabolite and phagocytosis of neighbouring cells^[44], but this local effect is weak compared to other

GDEPT modalities. The fusion of TK with the VP22 protein can amplify the effect by transferring the enzyme to surrounding cells^[45]. Ganciclovir-triphosphate is incorporated into the DNA and causes apoptosis in a cell cycle-dependent manner, but it can cause mitochondrial toxicity in normal hepatocytes if the expression of HSV-TK is not restricted to HCC cells^[46,47]. Apart from the therapeutic purpose, HSV-TK can be considered a reporter gene for PET analysis. It has been successfully used to visualize transduction of HCC with adenoviral vectors in humans^[48]. So far, the good antitumor efficacy of the HSV-TK system observed in different animal models of HCC has not been demonstrated in the clinical setting^[42]. Clinical and pre-clinical studies performed on other cancers suggest that HSV-TK can act as an immunogen that cooperates in the establishment of a systemic or at least local response against the tumors^[49,50]. Nevertheless, combination with other therapies will be needed. In pre-clinical studies the radiation-inducible Egr-1 promoter was used to control the expression of HSV-TK in combination with radioisotopes (^{131}I lipiodol)^[51]. Thus, the expression of HSV-TK was stimulated by the internal radiation, and the antitumor effect of both treatments was synergistic.

The yeast Cytosine Deaminase converts the antifungal drug 5-fluorocytosine (5-FC) into the cytotoxic thymidylate synthetase inhibitor 5-fluorouracyl (5-FU)^[52]. This metabolite can diffuse locally and cause a wider bystander effect than phosphorylated ganciclovir, but the cytotoxicity is also cell cycle-dependent. The system has been used in animal models of primary and metastatic liver cancer with good results^[53,54]. The efficacy of 5-FU on HCC patients is very low, but this strategy could achieve high local concentrations of the drug. In this context, toxicity in normal liver should be carefully evaluated. In addition, the conversion of 5-FC to 5-FU by the cytosine deaminase present in habitual enterobacteria can contribute to toxicity^[55].

Other GDEPT approaches generate very potent DNA cross-linking agents whose effects are largely cell cycle-independent. These include the cytochrome P450/cyclophosphamide^[56] and the Nitroreductase/dinitrobenzamide CB systems. Palmer *et al*^[57] reported that the intratumor administration of a first generation adenoviral vector expressing Nitroreductase in HCC patients is safe and feasible. Transgene expression was dose-dependent and is supposed to be clinically relevant, although no pro-drug was administered to patients in this study. Strong immune responses against the vector and the therapeutic gene were observed, indicating that re-administration of the treatment may not be beneficial. Assessment of the antitumor effect and toxicity of this approach in patients receiving the pro-drug requires new clinical trials.

An approach closely related to GDEPT consists on the delivery of the sodium iodide symporter (NIS) gene to cancer cells^[58]. Since NIS is necessary for the internalization of ^{131}I in the cell, a higher dose is accumulated in cells expressing NIS, as happens in thyrocytes, resulting in cell cycle blockade and death. Using this method, the extent and location of gene transfer can be detected by tomography. An adenovirus vector

expressing NIS under the control of the CMV promoter has been used for the treatment of HCC in a model of chemically induced tumors in rats^[59]. After injection of the vector in pre-existing nodules, specific accumulation of ^{131}I and significant reduction in tumor volume was observed.

TARGETED EXPRESSION OF CYTOTOXIC/PRO-APOPTOTIC GENES

This strategy is based on the selective transfer of genes that will cause the destruction of the cancer cells by different mechanisms. The concept is similar to GDEPT, but in this case the effect does not depend on any exogenous drugs. This can be an advantage in some circumstances, but on the other hand it lacks the possibility of pharmacologically modulating the cytotoxicity. This means that the system relies mostly on the targeting of gene transfer and expression into cancer cells, using specific surface ligands or promoters. The promoters for α -fetoprotein (AFP) and TERT have been used to control the expression or the diphtheria toxin fragment A and other cytotoxic genes in HCC cells^[60,61], but the toxicity of these treatments in relevant animal models is unclear.

Alternatively, the mechanism of action of the lethal gene can provide some tumor specificity. This is the case for TNF-related apoptosis inducing ligand (TRAIL). Unlike other members of the TNF ligand family, such as FASL and TNF- α , TRAIL induces apoptosis preferentially on cancer cells and may have reduced hepatotoxicity^[62]. The extracellular domain of TRAIL works as a soluble cytokine (sTRAIL) and induces apoptosis of cancer cells at distant locations from the producing cell. In fact, an AAV vector expressing sTRAIL fused with a human insulin signal peptide has shown potent antitumor effect on subcutaneous liver cancer xenografts after oral or intraperitoneal administration of the vector^[63]. This systemic effect was achieved without significant liver toxicity. Other vectors developed for the expression of TRAIL include first generation and oncolytic adenoviruses with enhanced infectivity in cancer cells^[64,65]. Interestingly, the adenoviral E1A protein sensitizes cells to TRAIL-induced apoptosis^[66].

IMMUNOGENE THERAPY

The transfer of genes with the aim to elicit an immune response against tumors is one of the most extensively used strategies in the field of cancer gene therapy. It is based on the observation that cancer cells modify their characteristics and their environment in order to avoid being detected and rejected. If this can be reversed, the specificity and systemic nature of the immune system offers the possibility of controlling the primary tumor and block its dissemination, which is the ultimate goal of all oncologic treatments. The wide repertoire of immunogene therapy approaches can be grouped as follows.

Expression of immunomodulatory cytokines

Cytokines are key mediators in the function of the immune system. They have been extensively used to

stimulate the immune response against tumors, including interleukin 2^[67], 7^[68], 12^[69], 15^[70], 18^[71], 21^[72], 23^[73] and 24^[74]; interferon α ^[75], β ^[76], and γ ^[77]; tumor necrosis factor α ^[78]; granulocyte-macrophage colony stimulating factor (GM-CSF)^[79], and others. Their effects on different cell components of the immune system and their influence on the expression of endogenous factors are extremely complex. Most of these cytokines do not have an intrinsic tumor-specific effect, but they may enhance the precarious immune response against tumors if the dose, location and timing are carefully controlled. For example, interleukin-12 (IL12) promotes a T-helper cell type 1 (Th1) response with activation of cytotoxic T lymphocytes and natural killer cells (NK)^[80], together with an antiangiogenic effect^[81,82]. These effects are largely dependent on the induction of IFN- γ . The systemic administration of recombinant IL12 showed potential antitumor effects in humans^[83], but severe toxicity was observed^[84] and this modality of treatment was discarded. The use of gene therapy vectors enables the localization of IL12 expression to the tumor, especially if vectors with liver tropism such as those derived from adenovirus are used^[85]. The antitumor effect of this strategy on different animal models of HCC has been demonstrated by several groups^[86,87]. Tumor eradication and immunologic protection against relapse is achieved in a significant proportion of cases, including implanted tumors in syngenic animals and chemically-induced HCC in rats. These results led to a phase I clinical trial that demonstrated the safety and feasibility of intratumor injection of a first generation adenoviral vector expressing IL12 in primary and metastatic liver cancer patients^[88]. Using these vectors, the expression of IL12 was very low and transient. No complete responses were observed, but patients with HCC had a better outcome than other histological groups in this trial. Based on these results, improvements in the vectors are being investigated. The use of high-capacity adenoviral vectors carrying a liver-specific inducible system for the expression of IL12 allows the long-term expression of the cytokine in response to the inducer mifepristone. Using this vector, the levels and duration of cytokine expression can be modulated to achieve antitumor effect and avoid toxicity^[89]. Further improvement can be achieved by using a version of IL12 in which the p35 and p40 subunits are fused in a single protein using a short linker peptide^[90]. Experiments performed in rats bearing HCC indicate that the single chain IL12 is about 1000 times more potent than the native protein when an equivalent adenoviral vector is used to deliver the gene intratumorally^[87].

Other cytokines that deserve special attention are TNF- α and IL24 (also known as mda-7). These molecules have shown antitumor effect on animal models of HCC^[78,91], and ongoing clinical trials suggest the potential therapeutic effects on other malignancies in humans^[92,93]. IL24 is especially promising, because apart from its immune-regulatory activities it induces apoptosis preferentially in cancer cells^[94].

Taking into account the natural mechanism of immune response activation, pro-inflammatory cytokines and co-stimulatory signals should be combined to achieve an effective response and avoid anergy. It is possible that

the accessory signals are already present in the tumor, but there is evidence of enhanced antitumor effect when IL12 is transferred together with 41BB agonists^[95] or B7.1^[96] in animal models of HCC. The intratumoral injection of an adenoviral vector expressing CD40L achieved tumor eradication on a significant proportion of pre-existing HCC in a rat model^[97]. This molecule is normally expressed on activated T cells and interacts with CD40 on the surface of antigen-presenting cells.

A combination of different cytokines may be more effective and less toxic than the expression of a single cytokine at high levels. The injection of adenoviral vectors expressing IL12 and IP-10 (interferon- γ inducible protein-1) exerted a synergistic antitumor effect in a murine model of colon cancer when both molecules were expressed locally^[98]. This is in agreement with the "attraction and activation hypothesis", in which colocalization of immunostimulatory (IL12) and chemoattractant factors (IP10) is needed. Some pre-clinical data indicate that IL15 can increase the antitumor effect IL12 on HCC models^[99]. Interestingly, this could happen in the absence of IFN- γ function. Other combinations proposed for the treatment of HCC include IL12+GM-CSF^[100], IL12+MIP3 α ^[101], and IL21+IL15^[72]. An alternative to co-expression of individual cytokines is the construction of fusion proteins. You et al. used a retroviral vector to deliver an IL2/IL12 fusion gene and demonstrated enhanced survival of tumor-bearing rats compared with rats treated with the individual cytokines^[102]. The antitumor effect of cytokines can be enhanced by other gene therapy approaches like GDEPT using HSV-TK, as demonstrated by several groups that employed adenoviral or retroviral vectors for gene delivery in HCC models^[103,104].

Vaccination with tumor antigens and genetically modified cells

The transfer of genes encoding tumor-specific antigens such as AFP has been used with the aim to break the immune tolerance against HCC^[105]. The pre-clinical efficacy of this approach depends on the particular animal model employed^[106], suggesting that high variability could be expected in patients. A different approach consists on the administration of activated effector or antigen-loaded presenting cells to fight cancer. The efficacy of these cells can be increased if they are manipulated genetically to express antigens, cytokines or co-stimulatory molecules (*ex vivo* gene therapy). Syngenic fibroblasts or cancer cells expressing IL12^[107] or IL2 plus B7^[108] can trigger an immune response against HCC in murine models. However, the use of cancer cells as a source of antigens and cytokines poses obvious technical difficulties in the clinical setting. An attractive alternative is the use of autologous dendritic cells (DC), professional antigen presenting cells that express the co-stimulatory molecules (CD80, MHC class I and II, *etc.*) necessary for efficient activation of effector cells. DCs expressing AFP^[109], cytokines^[110] or co-stimulatory molecules^[111] have been successfully used in animal models of HCC and gastrointestinal cancer^[112]. These results encouraged the initiation of a phase I clinical trial in which DCs expressing IL12 after *ex-vivo* infection with an adenoviral

vector were injected into the tumor mass^[113]. However, it was demonstrated that the cells were unable to migrate to lymph nodes because they were sequestered into the tumor by local factors^[114], preventing an efficient activation of effector cells and the establishment of relevant antitumor immune responses.

Adoptive cell therapy consists on the infusion of autologous T cells or killer cells that have been expanded and activated *in vitro*. In animal models, it has been demonstrated that T cell expansion occurs *in vivo* in tumor-bearing mice that were treated with IL12^[115]. The infusion of these cells has antitumor effect on recipient mice, in synergy with *in vivo* gene therapy by an adenoviral vector expressing IL12. This suggests that immunogene therapy can be used in combination with adoptive T-cell therapy in order to increase the efficacy observed in clinical trials that used either strategy alone.

An important aspect that is becoming more relevant in recent years is the inhibition of regulatory signals that control the duration and intensity of the immune response, because this could enhance the efficacy of anticancer immunotherapy. For instance, the blockade of the B7-H1/PD-1 pathway by soluble PD-1 expression improved the immune response against an implanted HCC in mice^[116].

ANTI-ANGIOGENIC GENE THERAPY

The realization that tumor growth requires intense neo-vascularization is the basis for a series of approaches aimed to specifically block the cancer-induced formation of new vessels^[117]. Anti-angiogenic factors such as endostatin have been identified and have demonstrated the ability to inhibit tumor growth *in vivo*^[118,119]. This strategy is supposed to be safe because it does not affect the mature vessels of normal tissues. Since HCC is known to be much vascularized, antiangiogenic therapies may have a strong therapeutic benefit, probably in combination with other standard or experimental treatments. Gene therapy may play an important role in this field, because anti-angiogenic factors need to be delivered for long period of times to control the progression of tumors. In fact, an adenoviral vector carrying the endostatin cDNA was more effective than the direct injection of the protein^[120]. The combination of endostatin delivered by an AAV vector and chemotherapy (etoposide) achieved antitumor effect on metastatic liver cancer in mice^[121]. Of note, several strains of bacteria have been engineered as vectors to deliver endostatin into liver tumors. Bifidobacterium longum administered orally increased the survival of tumor-bearing mice^[122], whereas attenuated Salmonella choleraesuis accumulates in hypoxic tissues and has shown antitumor effect after intraperitoneal administration^[123].

Other anti-angiogenic approaches are focused on blocking the VEGF receptor, which is an important mediator of angiogenesis. This can be achieved by expressing the soluble form of VEGF receptor (KDR/Flk-1), which sequesters VEGF^[124]. The same approach has been used to block the endothelium-specific receptor Tie2, which affects direct tumor growth and neovascularization^[125]. The Pigment Epithelium Derived Factor (PEDF) has been recently discovered as an anti-

angiogenic protein expressed in normal liver^[126] that is downregulated in HCC patients, suggesting a possible role in tumor progression. The transfer of PEDF has antitumor effects in a murine model of HCC^[127]. NK4 is a fragment of the Hepatocyte Growth Factor (HGF) that acts as a HGF antagonist and blocks angiogenesis. The intrasplenic administration of an adenoviral vector expressing a secreted form of NK4 caused reduction in the vascularization and growth of pancreatic metastasis in the liver of mice^[128]. Finally, it should be mentioned that the inhibition of angiogenesis may be one of the most important mechanisms by which IL12 exerts its antitumor effect^[129].

ONCOLYTIC VIRUSES

Using the cytopathic effect of certain viruses to destroy cancer cells is an old idea, but the advances in viral vector design and production have renewed interest in the field of virotherapy. The objective is to obtain a virus that replicates and preferentially kills cancer cells, leaving the surrounding normal tissues relatively intact^[130]. This property is intrinsic to some viruses. For instance, Vesicular Stomatitis Virus (VSV), Measles Virus (MV) and Newcastle Disease Virus (NDV) are very sensitive to the inhibitory effects of IFN and replicate only in cancer cells that have developed mechanisms to counteract IFN pathways. Other viruses like reovirus replicate better in cells that present activation of the Ras oncogene^[131].

On the other hand, other viruses such as Adenovirus or HSV can be genetically modified to make their replication cancer-specific. One of the methods to achieve cancer specificity is the deletion of viral functions necessary for replication in normal cells, but not in cancer cells. For instance, the adenoviral protein E1A blocks pRB in the cell to force activation of the cell cycle, whereas E1B 55K blocks p53 to inhibit apoptosis at early times. Since both p53 and pRB pathways are commonly altered in cancer cells, adenoviruses lacking these functions will replicate preferentially in tumors^[132,133]. Another method to restrict the replication of viruses is to use tumor-specific promoters to control the transcription of viral genes important for replication, such as E1A and E4 for adenovirus^[134]. Parallel strategies have been used to achieve oncolytic herpes viruses^[135,136]. The control of the γ 134.5 gene expression determines the efficacy of HSV-1 replication in different cells, and the deletion of the ribonucleotide reductase function attenuates the virus in normal cells^[137].

An important property of oncolytic adenoviruses is the possibility of accommodating therapeutic genes and the ability to act as gene therapy vectors with the advantage of tumor-specific amplification of gene expression^[138]. These genes code for pro-drug converting enzymes, immunostimulatory cytokines or pro-apoptotic proteins that enhance the oncolysis and/or achieve a systemic effect.

The mutant dl1520 adenovirus (also called ONYX-015 or CI-1042 later on) was described in 1996 as the first oncolytic adenovirus^[139]. It contains a deletion in the E1B 55K gene that achieves preferential replication in

cancer cells by different mechanisms^[140]. Although recent advances have yielded viruses with improved potency and specificity, the experience accumulated with ONYX-015 in the laboratory and in the clinic has been extremely useful for the advance of the field. The virus has shown partial antitumor effect on murine models of HCC^[141], and clinical trials for other cancers indicate a potential benefit when used in combination with chemotherapy^[142]. In the case of liver cancer, a clinical trial on HCC patients showed no evident antitumor effect. The first dose of ONYX-015 was administered intravenously and then by direct ultrasound-guided intratumoral injection on d 2, 15, 16, 29 and 30^[143]. The rationale of this regime was to elicit an immune response against the virus that causes a local reaction in the tumoral site. In a separate phase II trial in patients with metastatic colorectal cancer the virus was administered intravenously, and only transient stabilization of the disease could be observed in some cases^[144]. When the virus was administered intratumorally in a different clinical trial for hepatobiliary tumors, transient reduction of tumor markers in serum (CEA, AC19-9 or AFP) was observed in 50% patients, although radiological responses were less than 10%^[145]. These results support the notion that ONYX-015 has limited therapeutic effect as monotherapy on HCC patients, especially if systemic routes are used. When the virus was administered intravenously in combination with 5-Fluorouracil and leucovorin in patients with liver metastases of gastrointestinal cancers, 25% of cases presented partial or minor (< 50%) radiological responses, with good tolerance and evidence of adenovirus replication in tumors^[146]. An independent trial in patients who had failed previous treatment with 5-FU suggested increased survival when the virus was injected in the hepatic artery in combination with 5-FU^[147]. Interestingly, an early radiological increase in tumor volume was attributed to virus-induced necrosis rather than tumor progression in several patients. This should be taken into account in order to evaluate efficacy and avoid removal of responding patients in clinical trials. To this end, PET can be a more reliable technique. Now clinical trials with a virus similar to ONYX-015 (H101) are being conducted in China. A phase III trial in squamous cell cancer patients showed increased response rate in combination with chemotherapy, and the virus has recently obtained approval in that country^[148].

Other oncolytic adenoviruses have been developed, and show promising results (usually better than ONYX-015) in animal models of HCC. However, their performance in clinical trials has not been tested so far. The AFP promoter was used to control the expression of the E1A viral gene, with or without E1B 55K deletion, and this achieved preferential replication in AFP-producing HCC cells^[149,150]. The same effect is observed in metastatic gastrointestinal cancer using a virus controlled by the CEA promoter^[151]. A broader cancer spectrum is achieved when other tumor-specific promoters such as human TERT^[152,153] and E2F-1^[154] are used. The efficacy of these agents can be increased if they are adapted as gene therapy vectors for therapeutic genes ("armed" viruses), because viral oncolysis usually cooperates with the effect of the gene. Oncolytic adenoviruses expressing GM-

CSF^[155], TRAIL^[65,156], Smac^[157], Cytosine Deaminase^[158] and endostatin^[159] have demonstrated better performance than the previous versions.

The field of virotherapy has been enriched by the incorporation of oncolytic agents derived from different viruses, which may solve some of the limitations observed with adenovirus. For instance, HSV-1 exerts a potent oncolytic effect and its large genome can accommodate different exogenous genes, apart from the endogenous TK^[160]. The complex genome of HSV-1 allows multiple modifications that can be exploited to achieve tumor specificity. The G207 mutant contains a disruption in the UL39 gene that eliminates the ribonucleotide reductase function and determines preferential replication in cancer cells^[161]. It is attenuated by deletion of a single copy of the ICP6 gene and both copies of the γ 134.5 neurovirulence gene, but efficient elimination of HCC cells has been reported^[162]. The NV1020 virus harbours a deletion over the joint region of the genome, but retains the ICP6 gene and one copy of γ 134.5^[163]. A clinical trial using this virus is ongoing for patients with gastrointestinal cancer metastatic to the liver. The rRp450 HSV-1 variant carries the cytochrome p450 gene as a pro-drug converting enzyme^[164]. This virus has shown promising antitumor effect on HCC models, although complete eradication of metastatic liver cancer was not observed after single or multiple intraportal administrations. In addition, significant antitumor effect has been obtained in liver cancer models using herpes virus expressing Cytosine deaminase^[165] or IL12^[166].

VSV-derived viruses are emerging as a new class of oncolytic agents. A single injection of a recombinant VSV virus into the hepatic artery increased the survival of rats bearing multifocal HCC, and multiple doses achieved long term survival and tumor eradication in nearly 20% of the animals^[167]. This strategy is being investigated in human patients. Interestingly, experiments performed in rats indicate that a prophylactic treatment with IFN- α reduces the toxicity of the virus on normal tissues and elevates its therapeutic index^[168].

CONCLUSION

The treatment of hepatic malignancies (both primary tumors and metastatic cancer of the liver) remains a challenge that needs new approaches. Gene therapy is an experimental discipline in continuous evolution that offers interesting opportunities for the treatment of liver cancer. Following early excitement about gene therapy possibilities, the field soon realized its limitations and is now systematically addressing fundamental issues to solve them. The transfer of genes to the majority of cancer cells is still unrealistic for solid tumors, even with the best vectors available to date. Immunogene therapy approaches try to circumvent this limitation and extend the antitumor effect to distant metastases. Pre-clinical studies have validated the concept, but at the same time the results in animal models reveal that the efficacy of immunotherapy is very limited in advanced liver cancer. Hence, it is not surprising that the results of early phase clinical trials are apparently deceiving. Oncolytic adenoviruses were envisioned as autonomous

therapeutic agents that would seek and destroy cancer cells, amplifying the initial load until the tumor is eradicated. Now we know that they find important physical barriers that limit their distribution inside the tumor. Moreover, the immune system will control the spread of the viruses in a few days and neutralize further administrations, leaving a narrow time frame for them to display their oncolytic activity. An additional obstacle for the clinical application of most gene therapy approaches is the cost and technical difficulties of large scale production of the vectors.

Despite all these difficulties, gene therapy may play an important role as an adjuvant to other standard or experimental treatments against liver cancer in the near future. There is evidence that different gene therapy approaches like GDEPT or oncolytic viruses have synergistic effects when combined with chemotherapy or radiotherapy. The different mechanisms of action favour these combinations and may prevent the development of resistance to the treatment. As the knowledge of tumor immunology advances, more rational immunogene therapy approaches are designed. In addition, the improvement of invasive techniques for locoregional treatment of HCC can be used to deliver gene therapy vectors inside the tumor, increasing their safety and efficacy.

In summary, gene therapy will improve the management of liver cancer patients in the future, probably as part of an individualized multimodal therapy. This will require close collaboration and a continuous flow of information between basic, applied researchers and health care professionals.

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EDITORIAL

Heavy smoking and liver

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Abstract

Smoking causes a variety of adverse effects on organs that have no direct contact with the smoke itself such as the liver. It induces three major adverse effects on the liver: direct or indirect toxic effects, immunological effects and oncogenic effects. Smoking yields chemical substances with cytotoxic potential which increase necroinflammation and fibrosis. In addition, smoking increases the production of pro-inflammatory cytokines (IL-1, IL-6 and TNF- α) that would be involved in liver cell injury. It contributes to the development of secondary polycythemia and in turn to increased red cell mass and turnover which might be a contributing factor to secondary iron overload disease promoting oxidative stress of hepatocytes. Increased red cell mass and turnover are associated with increased purine catabolism which promotes excessive production of uric acid. Smoking affects both cell-mediated and humoral immune responses by blocking lymphocyte proliferation and inducing apoptosis of lymphocytes. Smoking also increases serum and hepatic iron which induce oxidative stress and lipid peroxidation that lead to activation of stellate cells and development of fibrosis. Smoking yields chemicals with oncogenic potential that increase the risk of hepatocellular carcinoma (HCC) in patients with viral hepatitis and are independent of viral infection as well. Tobacco smoking has been associated with suppression of p53 (tumour suppressor gene). In addition, smoking causes suppression of T-cell responses and is associated with decreased surveillance for tumour cells. Moreover, it has been reported that heavy smoking affects the sustained virological response to interferon (IFN) therapy in hepatitis C patients which can be improved by repeated phlebotomy. Smoker's syndrome is a clinico-pathological condition where patients complain of episodes of facial flushing, warmth of the palms and soles of feet, throbbing headache, fullness in the head, dizziness, lethargy, prickling sensation, pruritus and arthralgia.

INTRODUCTION

Lighting a cigarette creates over 4000 harmful chemicals with hazardous adverse effects on almost every organ in the body. The impact of heavy smoking on the pathogenesis of liver disease and response to interferon therapy among chronic hepatitis patients has been overlooked. Before we begin this article; it is necessary to define who is a heavy smoker and to shed light on the common toxic constituents of cigarette smoking.

WHO IS A HEAVY SMOKER?

Heavy smokers are variably defined, some studies suggest exposure to two or more packets (≥ 40 cigarettes) a day for 10 years or more^[1]. On the other hand, Marrero *et al*^[2] have defined heavy smokers as those exposed to greater than 20 pack-years.

COMMON CONSTITUENTS OF CIGARETTE SMOKE

The constituents of smoke are contained in either the particulate phase or gas phase.

Particulate phase

Particulate phase components include tar, polynuclear hydrocarbons, phenol, cresol, catechol and trace elements (carcinogens), nicotine (ganglion stimulator and depressor), indole, carbazole (tumor accelerators)^[3], and 4-aminobiphenyl^[4].

Gas phase

Gas phase contains carbon monoxide (impairs oxygen transport and utilization), hydrocyanic acid, acetaldehyde, acrolein, ammonia, formaldehyde and oxides of nitrogen

(cilitoxin and irritant) nitrosamines, hydrazine and vinyl chloride (carcinogens)^[3].

ADVERSE EFFECTS OF SMOKING ON THE BODY

Smokers are at greater risk for cardiovascular diseases (ischaemic heart disease, hypertension), respiratory disorders (bronchitis, emphysema, chronic obstructive lung disease, asthma), cancer (lung, pancreas, breast, liver, bladder, oral, larynx, oesophagus, stomach and kidney), peptic ulcers and gastroesophageal reflux disease (GERD), male impotence and infertility, blindness, hearing loss, bone matrix loss, and hepatotoxicity^[5,6].

ADVERSE EFFECTS OF SMOKING ON THE LIVER

Beside the hazardous effects mentioned before; smoking causes a variety of adverse effects on organs that have no direct contact with the smoke itself such as liver. The liver is an important organ that has many tasks. Among other things, the liver is responsible for processing drugs, alcohol and other toxins to remove them from the body. Heavy smoking yields toxins which induce necroinflammation and increase the severity of hepatic lesions (fibrosis and activity scores) when associated with hepatitis C virus (HCV)^[7] or hepatitis B virus (HBV) infection^[8]. Cigarette smoking increases the risk of developing HCC among chronic liver disease (CLD) patients^[9] independently of liver status. Association of smoking with hepatocellular carcinoma (HCC) irrespective of HBV status has been reported^[10,11].

How does smoking affect the liver?

Smoking induces three major adverse effects on the liver: toxic effects either direct or indirect, immunological effects and oncogenic effects.

Toxic effects of smoking on the liver

Direct toxic effect: Smoking yields chemical substances with cytotoxic potentials^[12]. These chemicals created by smoking induce oxidative stress associated with lipid peroxidation^[13,14] which leads to activation of stellate cells and development of fibrosis. In addition, smoking increases the production of pro-inflammatory cytokines (IL-1, IL-6 and TNF- α) involved in liver cell injury^[15]. It has been reported that smoking increases fibrosis score and histological activity index in chronic hepatitis C (CHC) patients^[7] and contributes to progression of HBV-related cirrhosis^[8].

Indirect toxic effects (concomitant polycythemia)

Heavy smoking is associated with increased carboxyhaemoglobin and decreased oxygen carrying capacity of red blood cells (RBCs) leading to tissue hypoxia. Hypoxia stimulates erythropoietin production which induces hyperplasia of the bone marrow. The latter contributes to the development of secondary polycythemia

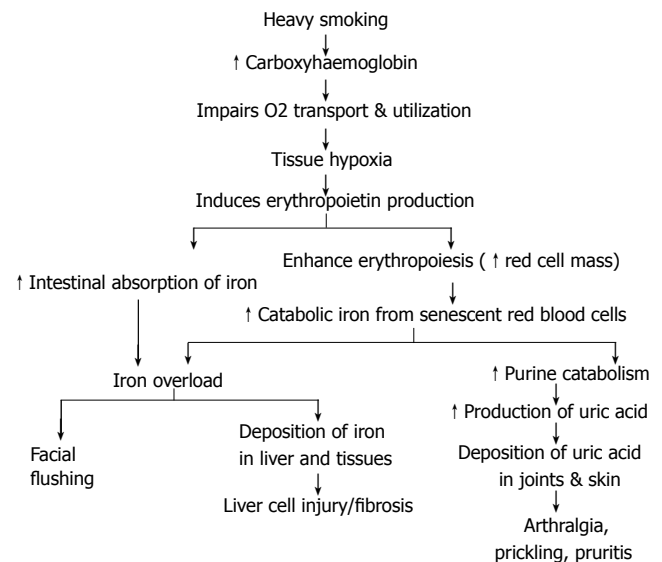


Figure 1 Development of smoker's polythemia and its adverse effects.

and in turn to increased red cell mass and turnover. This increases catabolic iron derived from both senescent red blood cells and iron derived from increased destruction of red cells associated with polycythemia^[16,17]. Furthermore, erythropoietin stimulates absorption of iron from the intestine. Both excess catabolic iron and increased iron absorption ultimately lead to its accumulation in macrophages and subsequently in hepatocytes over time, promoting oxidative stress of hepatocytes^[18]. Accordingly, smoking might be a contributing factor to secondary iron overload disease in addition to other factors such as transfusional haemosiderosis, alcoholic cirrhosis, thalassemia, sideroplastic anemia and porphyria cutanea tarda.

In the meantime, increased red cell mass and turnover are associated with increased purine catabolism which promotes excessive production of uric acid. Eventually uric acid is deposited in tissues and joints as manifested clinically by pricking sensation, pruritus and arthralgia^[19] (Figure 1).

Smoker's syndrome: Smoker's syndrome is a clinicopathological condition reported in patients smoking ≥ 40 cigarettes or 10 stones of popular shisha (water-pipe) in Egypt per day, over a long time. These patients suffer from episodes of facial flushing, warmth of the palms and soles, throbbing headache, fullness in the head, dizziness, lethargy, pricking sensation, pruritus and arthralgia^[20]. However, the majority of patients who smoke less than the described level are subject to biochemical changes rather than clinical manifestations.

Facial flushing, the most prominent symptom, is explained by capillary vasodilatation associated with increased blood flow through the skin. The vasodilatation may be attributed to the direct action of vasodilator constituents of the smoke as well as to excess haemoglobin saturation^[21] reported among heavy smokers^[20].

On examination of these smokers, the face appears dusky-red and/or pigmented, the pulse is full. The smokers suffer from hypertension, joint stiffness and swelling.

Some of them have experienced cerebrovascular and cardiovascular strokes. Laboratory studies have revealed an increased Hb level (> 160 g/L) and haematocrit (> 55 mL/100 mL) in almost all the patients and raised ALT (> 2 fold), uric acid (> 6 mg/dL), serum iron (> 165 μ g/dL) and ferritin in most of the patients. Histopathological examination reveals hepatic necro-inflammation, apoptotic necrosis, fibrosis, and deposition of iron in hepatocytes as demonstrated by Perl's stain.

IMMUNOLOGICAL EFFECTS OF SMOKING

Smoking affects both cell-mediated and humoral immune responses^[22]. Nicotine blocks lymphocyte proliferation and differentiation including suppression of antibody-forming cells^[15,23] by inhibiting antigen-mediated signaling in T-cells^[15,23,24] and ribonucleotide reductase^[25]. Furthermore, smoking induces apoptosis of lymphocytes^[26] by enhancing expression of Fas (CD95) death receptor which allows them to be killed by other cells expressing a surface protein called Fas ligand (FasL). Smoking induces elevation of CD8+ T-cytotoxic lymphocytes^[14], decreased CD4+ cells, impaired NK cell activity^[27] and increases the production of pro-inflammatory cytokines (IL-1, IL-6, TNF- α)^[15].

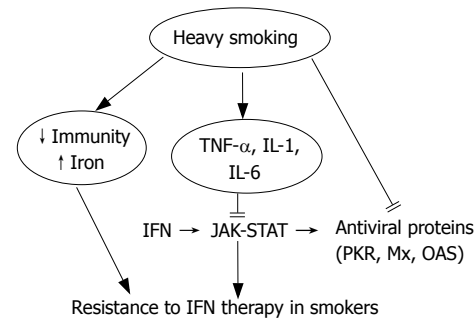
Although smoking has long-term adverse effects; cessation of smoking reversed these effects, such as elevation of NK activity which is detectable within one month of smoking cessation^[28], elevation of both antibody- and cell-mediated immune responses as well as decreased proinflammatory cytokines and increased antioxidant activity.

ONCOGENIC EFFECTS OF SMOKING

Smoking yields chemicals with oncogenic potentials such as hydrocarbons, nitrosamine, tar and vinyl chloride^[29]. Cigarette smoking is a major source of 4-aminobiphenyl, a hepatic carcinogen which has been implicated as a causal risk factor for HCC^[4]. Smoking increases the risk of HCC in patients with viral hepatitis^[9,30,31]. Furthermore, recent data from China and Taiwan have shown an association of smoking with liver cancer independent of HBV status^[10,11]. Tobacco smoking is associated with reduction of p53, a tumour suppressor gene^[32,33] which is considered "the genome guardian". Suppression of T-cell responses by nicotine and tar is associated with decreased surveillance for tumour cells^[25]. El-Zayadi *et al*^[20] reported that heavy smokers accumulate excess iron in hepatocytes which induces fibrosis and favours development of HCC. Smoking is considered a co-factor with HBV and HCV for hepatocarcinogenesis^[31]. In addition, suppressed mood, a common feature among heavy smokers, increases the risk for development of cancer^[25].

SMOKING AND LIVER CELL INJURY AMONG CHRONIC HEPATITIS C PATIENTS

El-Zayadi *et al*^[20] have reported an association between heavy smoking and liver cell injury in the form of necroinflammation, apoptosis and excess iron deposition



El-Zayadi A, 2005

Figure 2 Possible mechanisms of resistance to IFN- α therapy for viral hepatitis among heavy smokers.

in the liver. These effects are attributed to iron overload with consequent iron deposition in hepatocytes^[20,34]. Excess hepatic iron induces oxidative stress and lipid peroxidation^[13,14]. However, iron overload will not correct itself and the only exit of iron from the body is by bleeding or frequent chelation^[35]. Therapeutic phlebotomy allows excess iron to be removed from the body and chelation of labile iron from the liver.

SMOKING AND THE RESPONSE TO IFN THERAPY AMONG CHRONIC HEPATITIS C PATIENTS

El-Zayadi *et al*^[36] reported that smokers suffering from chronic hepatitis C tend to have a lower response rate to IFN therapy. Therapeutic phlebotomy among chronic hepatitis C patients improves the response rate to IFN therapy^[37,38]. Furthermore, the authors recommended that chronic hepatitis C patients should be advised to avert smoking before embarking on IFN therapy^[36].

Several mechanisms have been implemented in resistance to IFN therapy in heavy smokers which are summarized in Figure 2. First, heavy smoking causes immunosuppression^[22] such as reduction in CD4+ cells, impaired NK cytotoxic activity^[27] and recognition of virus-infected cells, and induces apoptosis of lymphocytes^[26]. Second, heavy smoking increases hepatic iron overload which is involved in resistance to IFN^[20]. Third, smoking induces pro-inflammatory cytokines (IL-1, IL-6, TNF- α)^[15] that mediate necroinflammation and steatosis. Fourth, smoking directly modifies IFN- α -activated cell signaling and action^[39].

The present article sends a message indicating that smoking is an underestimated risk factor for liver disease. In this respect, further well-designed studies are needed to clarify this issue.

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EDITORIAL

Recent trends in the epidemiology of inflammatory bowel diseases: Up or down?

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Abstract

Inflammatory bowel disease (IBD) is traditionally considered to be common in the Western world, and its incidence has sharply increased since the early 1950s. In contrast, until the last decade, low prevalence and incidence rates have been reported from other parts of the world including Eastern Europe, South America, Asia and the Pacific region. Recent trends indicate a change in the epidemiology of IBD with previously low incidence areas now reporting a progressive rise in the incidence, while in West European and North American countries the figures have stabilized or slightly increased, with decreasing incidence rates for ulcerative colitis. Some of these changes may represent differences in diagnostic practices and increasing awareness of the disease. The quality of studies is also variable. Additional epidemiologic studies are needed to better define the burden of illness, explore the mechanism of association with environmental factors, and identify new risk factors.

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Key words: Inflammatory bowel diseases; Ulcerative colitis; Crohn's disease; Incidence; Epidemiology

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INTRODUCTION

The pathogenesis of ulcerative colitis (UC) and Crohn's disease (CD) is only partly understood. Inflammatory bowel disease (IBD) is a multifactorial disease with probable genetic heterogeneity. In addition, several environmental risk factors contribute to the pathogenesis. During the past decades, the incidence pattern of both diseases has

changed dramatically, showing some common but also quite distinct characteristics of the two disorders. Differences in geographic distribution, and particularly changes in incidence over time within one area, may provide insight into possible etiological factors^[1].

Several studies have been conducted on the epidemiology of IBD^[1-3]. The geographical incidence of IBD varies considerably. The highest incidence rates are traditionally reported in Northern and Western Europe as well as North America, whereas lower rates are recorded in Africa, South America and Asia, including China^[2]. It is more common in developed, more industrialized countries, pointing at urbanization as a potential risk factor. The incidence rate of UC varies greatly between 0.5-24.5/10⁵ inhabitants, while that of Crohn's disease varies between 0.1-16/10⁵ inhabitants worldwide, with prevalence rates of IBD reaching up to 396/10⁵ inhabitants. Recent data from South Europe^[3], East Europe^[4] and Asia^[5] in the mid-1990s report a rise in incidence values in some areas already comparable to rates reported from Northern Europe or North America. The gap between areas with conventionally high and low incidence rates is diminishing. A further difference is that the previously reported predominance of UC is diminishing, as CD is becoming more prevalent^[6].

INCIDENCE IN THE WESTERN WORLD: NORTH/WEST EUROPE AND NORTH AMERICA

The first assessment of the incidence of IBD was carried out retrospectively in Rochester, Minnesota^[7]. The reported incidence of Crohn's disease was 1.9/10⁵ in 1935-1954. The incidence rates began to increase in the late 1930s in the United States and in the 1950s in North and Western Europe. From the 1960s onwards, an increasing number of studies of Crohn's disease and ulcerative colitis have been published. Most of these studies investigated the incidence retrospectively over rather short time periods and in small populations. Many of these studies were limited by incomplete case ascertainment.

The increase in the incidence of ulcerative colitis precedes the increase in the incidence of Crohn's disease by about 15-20 years. In the late 1990s, the incidence of UC leveled off to a plateau or even decreased^[6], while the incidence of CD was still increasing in most European countries. Recent data, however, suggest a further increase in the incidence of IBD, at least in some North European countries. The prevalence of Crohn's disease in North America ranges from 26.0 to 198.5 cases per 10⁵ persons.

The incidence rates range from 3.1 to 14.6 cases per 10^5 persons^[1,7]. That of UC varied between 6.0 and 14.3 from 1981 to 1994. Both ulcerative colitis and Crohn's disease appear to be more frequent in the northern parts of the US than in the south.

Similarly, previous studies in Northern and Western Europe in the 1970s and 1980s suggested that the incidence is decreasing from north to south^[8-11], but in the early nineties, the European IBD Study Group found comparable rates between Southern and Northern Europe^[3]. During the period between October 1991 and October 1993, twenty European centers in twelve countries prospectively collected all newly diagnosed IBD patients, in a population-based manner using a standard protocol for case ascertainment. The overall incidence was $10.4/10^5$ for ulcerative colitis (northern centers: $11.8/10^5$ vs southern centers: $8.7/10^5$) and $5.6/10^5$ for Crohn's disease ($7.0/10^5$ vs $3.9/10^5$). For UC, the highest incidence was found in Iceland and the lowest in southern Portugal. For CD, the highest incidence rates were observed in the southeastern Netherlands and in northwestern France. Nonetheless, the excess in northern centers was less pronounced than previously reported. Similarly, gradually increasing incidence values were reported from Italy between 1978 and 1992 (from $3.8/10^5$ to $9.6/10^5$ for UC and from $1.9/10^5$ to $3.4/10^5$ for CD) in the well-known Florence study^[8]. In addition, in recent publications, the incidence rate of UC in Central Greece has been reported to be as high as $11.2/10^5$, similar to that observed in high incidence North European centers^[9].

In addition, we have to note that continuously increasing incidence values are still reported from some high incidence areas, e.g. Denmark and Sweden. Earlier studies have reported an incidence of $4.1/10^5$ for CD and $9.2/10^5$ for UC from the 1980s^[10], while recent data in 2003-2005 indicate even higher incidence rates (CD: $8.6/10^5$, UC: $13.4/10^5$)^[11]. In Stockholm, the corresponding incidence data for CD in 1990-2001 were $4.9/10^5$ in 1985-89 and $8.3/10^5$ in 1990-2001^[12]. In contrast, data from the UK indicate a plateau with an incidence around $10.0/10^5$ - $13.9/10^5$ for UC, while it is still increasing ($3.9/10^5$ - $8.3/10^5$) in CD^[13].

A further difference is that the previously reported predominance of UC is diminishing, as CD is becoming more prevalent. Furthermore, decreasing incidence values in 1988-1999 were reported from Northern France. The incidence of CD was increased slightly from $5.2/10^5$ - $6.4/10^5$, while that of UC decreased from $4.2/10^5$ to $3.5/10^5$ ^[12].

INCIDENCE OF IBD IN OTHER PARTS OF THE WORLD: EAST EUROPE, ASIA AND SOUTH AMERICA

Until recently, only limited data was available on the epidemiology of IBD from other parts of the world. Most of these data are retrospective, hospital based surveys. One of the few exceptions is the study by Vucelic *et al*^[14] from Croatia, who conducted a prospective study on the incidence of IBD in Zagreb in 1980-1989. The study was population-based, including inpatient and outpatient data, as well as general practitioners' reports. The reported

incidence rate is $1.5/10^5$ inhabitants for UC and $0.7/10^5$ for CD. The prevalence of UC and CD was $21.4/10^5$ and $8.3/10^5$ respectively at the end of 1989.

In the last few years, a significant number of papers and reviews have been published on the epidemiology of IBD from Eastern Europe and Asia^[4,5]. However, there is still a significant lack of data from Latin America, Africa and Australia. Most of these studies have reported a change in the epidemiology of IBD. The rise in incidence began in the early 1990s, in parallel with changes in governing systems and social environment. In Eastern Europe, a population-based epidemiology survey from Hungary^[15] reported a sharp increase in incidence of UC from $1.7/10^5$ during 1977-1981 to $11.0/10^5$ during 1997-2001. In CD, a similar trend was observed (from $0.4/10^5$ to $4.7/10^5$). The ratio of UC/CD incidence rates decreased from 4.0 to 2.3 during the observed periods. The prevalence of UC and CD was $142.6/10^5$ and $52.9/10^5$ inhabitants respectively at the end of 2001. Similarly, in a prospective study Mijandrusic Sincic *et al*^[16] reported much higher rates in Croatia ($7.0/10^5$ for CD and $4.3/10^5$ for UC) in 2000-2004 compared to previous reports.

In contrast, a questionnaire-based nationwide epidemiological survey^[17] from Romania, over a period of one year between June 2002 and June 2003, still reported low incidence of both UC ($0.97/10^5$) and CD ($0.50/10^5$). Corresponding prevalence data were also very low ($2.25/10^5$ in UC and $1.51/10^5$ in CD). Similarly, low incidence was reported in a population-based prospective study from Estonia^[18]. The data were collected between 1993 and 1998 in Tartu country (151301 inhabitants) from the internal medicine, paediatrics and surgery departments. A total of 16 UC and 13 CD patients were diagnosed, equalling an average incidence of $1.7/10^5$ for UC and $1.4/10^5$ for CD. However, the relative small area and small absolute number of patients might have biased the data.

Similar trends in UC have been observed in Asia. Although both incidence and prevalence rates of IBD are still low compared with Europe and North America, they are rapidly increasing. An emergence of UC is apparent. In contrast, the incidence of CD is still low. An analysis of UC patients from China reported a total of 10 218 cases from 1981 to 2000^[19]. Of these, 2506 were diagnosed between 1981 and 1990, and 7512 between 1991 and 2000, a three-fold increase. The most recent review^[11] of Chinese literature reveals that 143 511 cases of IBD (140120 of UC and 3391 of CD) were described during the last 15 years, with an 8.5-fold increase during the last 5 years compared with the first 5 years. In addition, though figures are still low, Leong *et al*^[20] reported a three-fold increase in the incidence of CD in the Chinese population of Hong Kong, from $0.3/10^5$ in 1986-1989 to $1.0/10^5$ in 1999-2001. A hospital-based study came to the same conclusions. An estimated incidence of $0.28/10^5$ and a prevalence of $1.38/10^5$ were reported between 1950-2002 from 22 provinces of China^[21].

Recent reviews from Japan also report an increase in the incidence and prevalence of IBD. Hospital-based investigations reported that the incidence of UC was $1.95/10^5$, and the prevalence was $18.12/10^5$ in 1991^[21]. In a retrospective study from Seoul, South Korea, the

prevalence of UC was $7.57/10^5$, with a significant increase in incidence rates from $0.20/10^5$ in 1986-1988 to $1.23/10^5$ in 1995-1997^[21]. In Thailand, UC is still uncommon, with only 40 cases reported between 1988 and 2000^[22].

Singapore is also witnessing a higher prevalence of IBD, recently estimated at $6/10^5$ ^[23]. The current prevalence of UC is $17/10^5$, whereas that of CD is lower, at $3.6/10^5$. The impact of ethnicity in various Asian populations has also been studied in Singapore, a multiracial city-state composed of Chinese, Malay and Indian groups^[24]. The prevalence of UC in the three races has been calculated to be $6.2/10^5$ for Chinese, $4.8/10^5$ for Malay and $16/10^5$ for Indian subjects. Similar prevalence data were reported from a recent retrospective hospital-based study from Kuala Lumpur, Malaysia. The prevalence of UC was $17.9/10^5$ in Indians, followed by $11.2/10^5$ in Chinese and only $3.7/10^5$ in Malays between 1985 and 1998. No such marked difference was seen in CD. The prevalence rates in Chinese, Malays and Indians were $4.0/10^5$, $2.9/10^5$ and $4.9/10^5$, respectively^[23].

The lower prevalence and incidence of IBD in Asia compared with the West are not universal. A population-based study from Northern India found that the prevalence rate of UC is $42.8/10^5$ ^[25]. This unexpectedly high prevalence has been recently confirmed by another population-based study in Punjab, also in Northern India, revealing a prevalence of $44.3/10^5$ and an incidence of $6.02/10^5$ ^[26]. However, a major limitation of these studies is that they only screened a population sample of around 22 000 and 52 000 inhabitants, respectively. Thus, the results rely on a very small number of definite cases extrapolated to the whole population of the area.

IBD in Middle-East is traditionally reported to be high among Jews from the United States and Northern Europe. In Israel, the incidence is somewhat lower and Ashkenazi Jews have a higher incidence than Sephardic Jews. In 2000, Niv *et al*^[27] reported an annual incidence of $5.04/10^5$ for UC for a ten-year follow-up period between 1987-1999. The prevalence rate rose from $121.0/10^5$ to $167.2/10^5$.

In contrast, Arab countries in the Middle East still report low incidence rates. A prospective hospital-based study from Saudi Arabia^[28] reported an estimated incidence of $0.5/10^5$ and prevalence of $5.0/10^5$ for IBD in children in 1993-2002. Similarly, a retrospective study from the same region estimated a $0.94/10^5$ mean incidence of CD in 1983-2002, with a gradual increase in incidence from $0.32/10^5$ to $1.66/10^5$ ^[29]. In contrast, in Turkey the incidence of UC was as low as $0.59/10^5$ - $0.69/10^5$ between 1998 and 2001, in a hospital-based study^[30]. Finally, a recent case series from Teheran, Iran suggests very low incidence rates, since authors reported that only 448 IBD patients were referred to or diagnosed in two university hospitals and two private GI clinics between 1992 and 2002^[31].

Only limited data are available from Central and South America. A recent prospective study has reported the incidence of IBD in Puerto Rico^[32]. The total incidence of IBD increased significantly between 1996 and 2000 ($3.07/10^5$ to $7.74/10^5$), being significantly higher than CD. In contrast, much lower incidence rates were reported from a population-based survey from Panama and

Argentina^[33] in 1987-1993. The annual incidence of UC was $1.2/10^5$ in Panama and $2.2/10^5$ in Argentina, with only a single case of CD being identified. Similarly, the incidence of both UC and CD admissions is low in Brazil with only 257 new cases (126 CD and 131 UC) diagnosed in 1980-1999^[34].

Finally, Gearry *et al*^[35] have recently reported one of the highest incidence rate of CD from New Zealand in 2004. The reported incidence of CD is $16.5/10^5$ and the incidence of UC is lower ($7.6/10^5$). Corresponding prevalence rates are $355.2/10^5$ and $145.0/10^5$.

INFLAMMATORY BOWEL DISEASE IN CHILDREN AND ELDERLY

It is important but difficult to study the epidemiology of IBD in children. Although both UC and CD are rare below the age of 11 years, the incidence of these diseases increases rapidly after adolescence. In the pediatric age group, several epidemiologic studies have been published with some evidence suggesting that the incidence of IBD (in particular, CD) has increased over the last ten years.

The explanation for the differences in incidence between studies is further complicated by different definitions of childhood. The upper age limit varies between 14 and 17 years of age. Furthermore, upper gastrointestinal involvement is reported to be more common in children with CD. In addition, we have to note that new diagnostic tools may lead to overinterpretation of IBD emergence, since the clinical importance of minute lesions such as small erosions diagnosed during capsule endoscopy or double-balloon enteroscopy, which may be often diagnosed as IBD in children with abdominal pain, is still to be determined. To exclude this possibility, new uniform criteria have been developed for the diagnosis of IBD in children by ESPGHAN^[36].

Most of the published data come from the United States and Western Europe. The largest prospective study from the United Kingdom and Ireland has reported a well-characterized cohort of 739 children with IBD^[37]. The mean incidence of CD and UC is $3.0/10^5$ and $1.5/10^5$. Higher values were reported in a prospective study from Wisconsin, United States between 2000 and 2001^[38]. The mean incidence of CD and UC is $4.5/10^5$ and $2.1/10^5$, respectively.

A further population-based study from Copenhagen^[39] reported that 7% of the 1161 patients diagnosed with UC and 6% of the 373 patients with CD had an onset before 15 years of age. The median age at diagnosis was 12 years (range 0-14 years), indicating a steep increase in incidence around puberty. During the 26 years of the study, no significant change in incidence was found, the mean incidence of IBD was $2.2/10^5$ (UC: $2.0/10^5$ and CD: $0.2/10^5$). In another study of a large area of Sweden, Lindberg *et al*^[40] prospectively studied all patients below 16 years of age with a diagnosis of definite or probable UC and CD in 1984-1995. An increase in the incidence of UC was found (from $1.4/10^5$ to $3.2/10^5$), but only in the age group of 11-16 years, not in early childhood. The incidence of CD did not change over the time studied.

In contrast, other studies have reported a marked increase in the incidence of pediatric IBD. A slight increase in the incidence of juvenile onset CD was reported from Scotland between 1981 and 1995 with marked differences in the incidence of CD between north and south^[41]. The incidence of UC and CD increased over the observed period in both south (UC: 0.9-1.6/10⁵ and CD: 1.8-2.5/10⁵) and north (UC: 0.9-1.8/10⁵ and CD: 2.8-3.5/10⁵) territories. Similarly, an increase in the incidence of CD was reported in a prospective study from northern Stockholm between 1990 and 2001^[42]. The incidence in CD rose from 1.5/10⁵ to 8.4/10⁵ (mainly in the age group of 10-15 years), while that of UC was relatively stable at approximately 1.8-1.9/10⁵, except for 1990-1992. One of the largest studies has been reported from Northern France^[43]. In a prospective follow-up study, a total of 7066 patients were diagnosed, 509 (7.2%) of them were under the age of 17 years at the time of diagnosis. During follow-up, a trend for an increase in the incidence of CD was observed (from 2.1/10⁵ to 2.6/10⁵), while the incidence of UC remained unchanged (0.8/10⁵).

In Eastern Europe, Pozler *et al*^[44] have recently published the results of a retrospective study investigating the incidence of CD in the Czech Republic in children diagnosed under the age of 15 years between 1990 and 2001. A marked increase in the incidence of CD was reported from 0.25/10⁵ in 1990 to 1.25/10⁵ in 2001.

The same trend has been reported from Melbourne, Australia^[45] in a retrospective, hospital-based study in children diagnosed under the age of 16 years. The incidence of CD rose from 0.13/10⁵ to 2.0/10⁵ between 1971 and 2001.

An interesting question is the incidence of IBD in elderly populations. At least according to the published data from Western Europe, it may not be as uncommon as previously suspected. A prospective Belgian study^[46] between 1993 and 1996 reported that the incidence of IBD in the elderly population (age at diagnosis > 60 years) is high (UC:4.5 and CD:3.5), was comparable to the figures for younger age groups (UC: 3.4 and CD: 4.8).

Similarly, comparable incidence rates for CD have been reported from France between 1994 and 1997^[47]. The annual incidence rate is 2.5/10⁵, with a higher proportion of colonic disease.

HOW CAN WE EXPLAIN THE RECENT EPIDEMIOLOGICAL TRENDS?

The incidence of IBD varies greatly worldwide. Genetic and environmental factors are assumed to play a significant role in the aetiology of the disease. The role of genetic factors is supported by ethnic and familial differences as well as twin studies^[1,4], while the differences in incidence rates among various geographical areas suggest a role of certain environmental factors. It is known that the incidence differs among different ethnic groups living in the same geographic region. This fact is mostly observed in Asian countries^[5,24]. Similarly, the genetic predisposition may have led to the observation that the risk for IBD in Asian immigrants surpassed that observed in UK's native

population with an odds ratio of 6.1^[48]. In addition, disease phenotypes may also vary among different races, e.g. in a recent study from the United States^[49] reported that African American CD patients are more likely to develop upper gastrointestinal, colorectal or perianal disease, uveitis and sacroileitis compared to whites, while Hispanics are at higher risk of developing perianal disease or erythema nodosum, supporting a role of underlying genetic variations. Finally, the prevalence of identified genetic risk factors (e.g., NOD2 in CD) is also different in the different populations.

An important change in the incidence of IBD has taken place in the last few decades. In most of the Western Europe countries, the incidence rate remains relatively stable or even decreased. In contrast, continuously increasing incidence rates of CD have been recently reported from other high incidence areas such as Denmark^[11] and Sweden^[12]. There is also an important change in the diagnosis of CD. A new classification system has been developed^[50], as well as new tools which are readily available for the investigation of the small-bowel (e.g., capsule endoscopy^[51,52] and double balloon enteroscopy^[53]), which may lead to a further increase in reported incidence rates. As a consequence, an unusually high upper gastrointestinal involvement (as high as 55%) has been reported in recent genetic studies in pediatric settings^[54]. A similar trend is also apparent in the adult cohorts. Nonetheless, it is important to note that the clinical importance of minute lesions still needs to be determined. Capsule endoscopic examinations due to abdominal pain can reveal small lesions (angiodysplasias and small erosions) in similar percentages of subjects^[55]. Since the penalty for false-positive testing is very high (unnecessary use of toxic and expensive medications, and the stigma of being diagnosed with a serious chronic disease), at present, capsule endoscopy and double balloon enteroscopy should be used only in limited clinical situations rather than routinely. In addition, the interpretation of microscopic disease, especially in children is rather controversial. Nonetheless, this still does not explain the higher incidence values reported in recent studies.

In previously low-incidence areas, especially in Eastern Europe^[4,15,16] and some regions in Asia^[26], the disease has become more prevalent. Until now, the role of private practice is limited in Eastern Europe and most Asian countries and the majority of patients are managed in the public health care system, enabling population-based epidemiological investigations. The diligent collection of data and case ascertainment is, of course crucial.

The increased incidence rates of both UC and CD observed in Eastern Europe and Asia raise further questions. What could be the cause of this change? In the 1970s and early 1980s the lower incidence rates could be partially explained by the use of fewer up-to-date diagnostic procedures (e.g., the relative low availability of selective enterography or colonoscopy). It is also possible that greater awareness, either by physicians or by patients, may result in the diagnosis of mild cases that might have been previously unnoticed. There has also been an important change in patients' behaviour, at least in Eastern Europe, as patients tend to seek medical advice more often and with milder

symptoms than they did two decades ago. In contrast, in Eastern Europe, due to various and multiple causes, the access to health care services has not improved much. The reported incidence rates of IBD in Hungarian^[15] and Croatian^[16] are in a similar range, as previously observed in Nordic countries^[3,56,57]. In contrast, other countries (e.g., Czech Republic, Poland, Romania and Slovakia) still report low incidence rates. These studies however, have several limitations. Some of them were retrospective surveys^[44], while others were based solely on the in- and outpatient records of a single hospital, extrapolating the results to the investigated area^[4]. Patients might have been unnoticed, thus the incidence rate is clearly underestimated. We believe that the increase in the incidence of IBD in Eastern Europe in the late 1980s and 1990s is real and not solely due to improved diagnosis, better health care access or more extensive search. This notion is also supported by the increase in more severe cases, which can only be interpreted as real.

The observed changes may be at least partially explained by the differences in study design. Most of the earlier studies from Eastern Europe, and still the majority of studies from Asia^[19,23], Middle-East^[29,30] and Central America^[33] are biased by methodological shortcomings. A great majority of them were retrospective, hospital-based surveys, which may have at least partly been responsible for the low incidence rates reported. In contrast, recent prospective, population-based studies, especially from Eastern Europe with extensive search for IBD cases^[25,32] have reported much higher incidence rates from the same regions with some exceptions^[20]. The situation is somewhat different in India. Though papers are prospective^[25,26], results might have been biased by methodological problems. Only questionnaire-based surveys were conducted and only a limited population sample was screened. Thus, the results rely on a very small number of definite cases extrapolated to the whole population of the area.

Diet, as a luminal antigen, is thought to be an important factor in the pathogenesis of IBD^[1,58]. In the last two decades, there has been a change in the lifestyle in Eastern Europe, Asia and Central America, as the standard way of living, including the diet, has become more "Westernized". This possibility is further supported by the differences in incidence and prevalence found within one region. In Eastern Europe^[4,15], the change in the incidence is parallel to the change in governing system and the prevalence is clearly different between the more "Western"-type living in the western regions, as opposed to the less rapidly changing eastern parts.

This raises the possibility that the prevalence of IBD, e.g., in the eastern parts of Europe and Asia is also capable of changing in the next one or two decades. An early signal of this change might be the reported increase in the incidence of UC in many of the Asian countries. Since the change in incidence of UC precedes that in CD in most countries, it is possible that different environmental factors are responsible for the increase in the incidence of UC and CD. Alternatively, the same environmental factors may act differently in the two diseases, resulting in different epidemiological patterns. This is supported by the recent trends in the incidence of UC in Asia and the similar changes in

the late 1980s and early 1990s in Eastern Europe, where the increase in the incidence of UC precedes that of CD. It is therefore, of outstanding interest to follow the temporal trends of IBD epidemiology in Asia, Eastern Europe and Central America. In addition, these studies offer a unique possibility to extensively study the role of possible environmental risk factors in the susceptibility of IBD.

Other possible environmental factors, such as perinatal events, childhood infections or measles have not been investigated outside North America and Western and Northern Europe^[59,60]. Measles vaccination is however universal in Eastern Europe making the disease very rare, in addition to a low birth rate. Early childhood hygiene is also well developed, supporting a possible role of the "oversheltered child" theory^[61]. The hygiene hypothesis suggests that skewing of the Th1/Th2 balance in early life is a major cause for the recent increase in allergic and autoimmune diseases. Recent data from different regions of Canada have controversial results^[62,63]. Nonetheless, since early and high level of childhood hygiene has existed, at least in Eastern Europe since the early 1970s, it does not explain the epidemiological trend observed in the late 1990s. In addition, the reported prevalence of other known environmental factors (e.g., smoking, frequency of appendectomy, contraceptive and use of NSAID and antibiotics) did not change significantly in the last decades, thus it is unlikely that these factors are essential in evolution of the epidemiology trends.

The incidence of IBD varies greatly worldwide. In traditionally high incidence areas, e.g. in West European and North American countries the figures have stabilized or slightly increased, with even decreasing incidence rates for ulcerative colitis. In contrast, low prevalence and incidence rates have been reported from other parts of the world including Eastern Europe, South America, Asia and the Pacific region. Recent trends however, also indicate a change in the epidemiology in these countries, since previously low incidence areas are now reporting a progressive rise in the incidence. Some of these changes may represent differences in diagnostic practices and increased awareness of the disease. The quality of the surveys is also variable. Most probably, inadequately identified environmental factors or a combination of these factors may be responsible for the recent rapid changes. Additional epidemiologic studies are needed to better define the burden of illness, explore the mechanism of association with environmental factors, and identify new risk factors.

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Elevated risk for gastric adenocarcinoma can be predicted from histomorphology

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Abstract

The number of patients with gastric cancer has more than doubled since 1985 in developing countries. Thus, the questions of whether it can be predicted from gastritis morphology, who is at risk and who has a lower risk of developing gastric carcinoma are raised. *H. pylori*-infection leads to erosions, ulcerations, carcinoma, mucosa associated lymphoid tissue (MALT)-lymphoma and extragastric diseases only in some individuals. The frequency of ulcerations among *H. pylori*-infected individuals is estimated to be 13%, gastric cancer about 1% and MALT lymphoma around 0.1%. In the literature a multistep model from chronic active *H. pylori*-infection through multifocal atrophy, intestinal metaplasia, dysplasia (intraepithelial neoplasia) and carcinoma has been described. But this model cannot be applied to all routine cases. Since risk factors such as metaplasia and atrophy are precancerous rather than precancerous conditions, this raises the question whether there is a better morphological marker. Differences in topography, grade and activity of *Helicobacter* gastritis in the antrum and corpus might be good markers for identifying those who are at risk of developing gastric cancer. It is known that the so-called corpus dominant *H. pylori* gastritis is found more frequently among individuals with early and advanced gastric cancer and within high risk populations. This is valid both for first-degree relatives of gastric cancer patients and for patients with gastric adenoma and hyperplastic polyps. In conclusion, corpus-dominant *H. pylori* gastritis is significantly more common in patients with advanced and early gastric cancer, first-degree relatives of patients with gastric cancer, patients with gastric adenoma and gastric hyperplastic polyps. Therefore, all these patients are at risk of developing gastric cancer. Next, the question of who is at risk of developing corpus-dominant gastritis is raised. It appears that patients with a low acid output more frequently develop gastric cancer. Eradication therapy is never performed too early but probably sometimes too late after the patients pass a "point of no return". Large prospective long term studies

are necessary to prove this and identify new reliable markers for gastric cancer development.

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Key words: *H. pylori*; Corpus-dominant helicobacter gastritis; Low acid output; Gastric adenocarcinoma; Histomorphology

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INTRODUCTION

H. pylori is recognized as the major pathogenetic factor in chronic active gastritis. Chronic active gastritis not only leads to gastric inflammation in various degrees but also can be the cause of gastric and duodenal ulcer disease and might give rise to gastric mucosa associated lymphoid tissue (MALT) lymphoma (marginal B-cell lymphoma). Interestingly, low grade MALT lymphoma regresses after antibiotic treatment for *H. pylori* eradication in a high percentage of cases.

HISTORICAL BACKGROUND

Prior to the morphological identification of *H. pylori* as the causative agent, gastric inflammation had already been considered a risk factor for gastric carcinoma. Correa postulated in 1988^[1] that chronic gastritis may lead *via* a multistep process to intestinal metaplasia and atrophy as an additional morphological risk factor for the development of carcinoma since these are frequently found to be closely related to the intestinal type of gastric cancer. In addition, today it has become clear that at least 50% of all cases of autoimmune atrophic gastritis which is considered to some varying degree as a precancerous condition, are probably induced by *H. pylori*^[2].

EPIDEMIOLOGICAL EVIDENCE

Mortality of gastric cancer is a significant burden not only on patients but also on the whole health system. There are regional differences depending on the geographical location (Table 1). For example, about 360 000 deaths

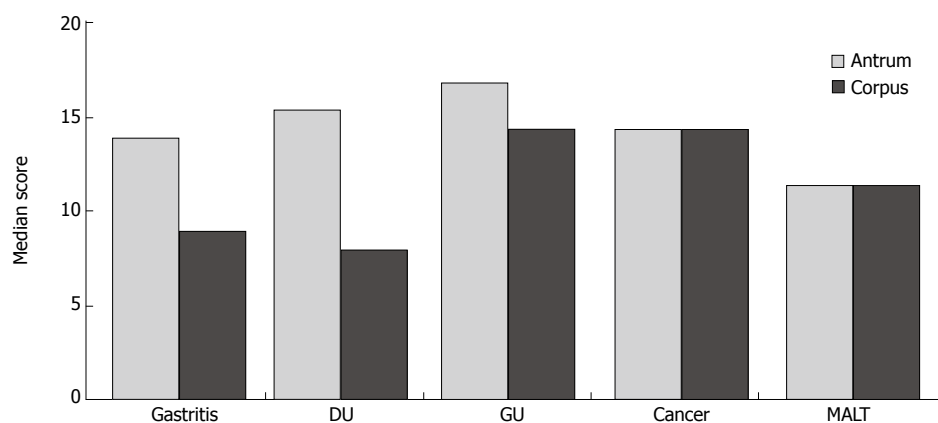


Figure 1 Median gastritis scores in antrum and corpus mucosa of patients with chronic active *H pylori* gastritis alone, duodenal ulcer (DU), gastric ulcer (GU), gastric cancer or MALT lymphoma ($n = 50$) (Modified from Meining *et al*^[16]).

Table 1 Gastric cancer mortality in different populations (Modified from Winawer *et al*^[31])

Countries	Yr	Yr
	2000	2020
Developed	334.000	440.000
Developing	543.000	983.000

occur due to gastric cancer each year in China with the world largest population^[3]. This means that every 2 min one patient dies of gastric cancer in China.

The knowledge on a pathogenetic association between gastric carcinoma and *H pylori* infection is mainly based on retrospective studies by observing resected stomachs. *H pylori* gastritis is usually present in gastric mucosa adjacent to cancerous lesions. Furthermore, a statistically significant correlation between the rate of infection and the incidence of gastric cancer has been observed in a number of populations, although in some populations, especially in Africa, such a correlation is not found^[4]. Thirdly, case-control studies demonstrated that the risk of developing gastric carcinoma is significantly higher in the presence of *H pylori*. Such an association is only described for the distal stomach but not for carcinomas in the proximal stomach, that may arise due to an increasing number of Barrett's esophagus^[5,6]. Many of these studies have methodological problems and weakness of data strength. The situation has improved nowadays. At least some prospective studies are available showing that if certain morphological features are present the patients are at risk of developing gastric carcinoma.

In Germany with 80 million inhabitants, more than 26 000 patients suffer from gastric carcinoma^[7]. Approximately 50% of them will not survive for 5 years, which underlines the importance of this disease for the health system.

WHO has classified *H pylori* as a class 1 carcinogen for the development of gastric carcinoma, but only a minority of individuals infected with *H pylori* develop gastric carcinoma. It is not clear up till now how the complex interaction occurs between host development of neoplasia in some patients but not in the majority of all others. It has long been known that nutritional factors, high salt intake, smoked meat, few vitamins might increase the risk

of gastric carcinoma^[8]. From our routine practice we know that cancer develops very rarely in normal gastric mucosa. It is of particular interest to identify risk markers capable of predicting the risk of developing gastric carcinoma. Healing of such high risk gastritis can then decrease the risk of developing gastric adenocarcinoma. Before *H pylori* was re-introduced into medicine as the causative agent for gastritis, it has been known that the presence of multifocal atrophic gastritis with intestinal metaplasia is a risk factor for gastric carcinoma^[1]. Interestingly, the diffuse (signet ring cell) type of carcinomas is not covered by such a hypothesis that gastric cancer might develop even in normal gastric mucosa, especially in very young adults, indicating a genetic background in those patients.

MORPHOLOGICAL FEATURES OF GASTRITIS WITH HIGHER RISK OF DEVELOPMENT OF ADENOCARCINOMA

Initial investigations on the topographic aspects of *H pylori* showed that *H pylori* colonization is less frequent and activity is less marked in the gastric corpus compared to the gastric antrum^[9-12]. Recent studies including the gastric cardia also suggest that *H pylori* colonization is denser in the gastric cardia than in the gastric corpus, in turn leading to more pronounced gastritis in this region^[13]. Nowadays it is well accepted that *H pylori* colonizes in the whole gastric mucosa. The infection induces chronic active inflammation anywhere in the gastric mucosa. An important task is therefore, to search for bacteria- and host-related factors that favor the development of gastric carcinoma. The results of studies carried out by our group at the end of the 1980s^[14] have prompted us to carry further studies in this field.

Comparison of gastritis scores in antrum and corpus biopsies

In a matched control study, our group has compared the gastritis score^[15] of individuals with NUD, duodenal ulcer, gastric ulcer, MALT-lymphoma and gastric carcinoma. These studies show that patients with chronic active *H pylori* gastritis, gastric ulcer and duodenal ulcer have significantly lower scores in the corpus than in the antrum, compared to cases of gastric cancer and MALT-lymphoma (Figure 1)^[15], but the scores are significantly lower in MALT lymphoma than in gastric cancer.

Table 2 Gastric cancer risk index in patients with active *H. pylori* gastritis

Grade of gastritis (infiltration by lymphocytes and plasma cells) is more severe in the corpus compared to the antrum or at least equally distributed
Activity of gastritis (infiltration by neutrophilic granulocytes) is more severe in the corpus compared to the antrum or at least equally distributed
Intestinal metaplasia present in the antrum and/or in the corpus

Each feature is scored with one point. Values between 0 and 3 and a total sum of 3 are possible (Adapted from Meining *et al.*^[16]).

Table 3 Prevalence of *H. pylori*-associated high risk gastritis (3-index-points) in relation to age (*n* = 845) (Modified from Leodolter *et al.*^[19])

Age	<i>n</i>	3-index-points
< 35 yr	114	0.9%
35-54 yr	116	2.7%
55-64 yr	186	6.5%
65-74 yr	145	12.7%
> 74 yr	83	12.6%

Comparison of antral and corpus gastritis in patients with gastric carcinoma or duodenal ulcer

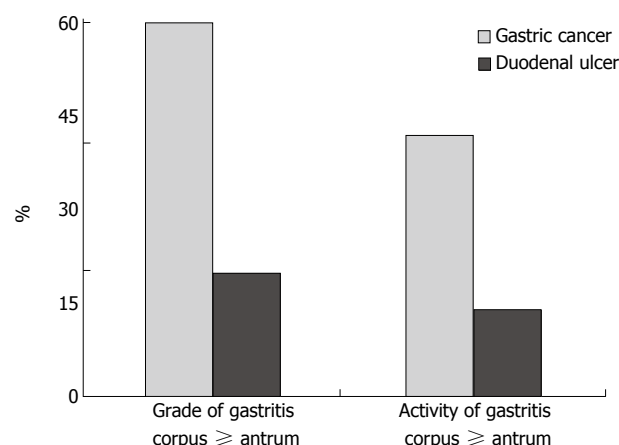
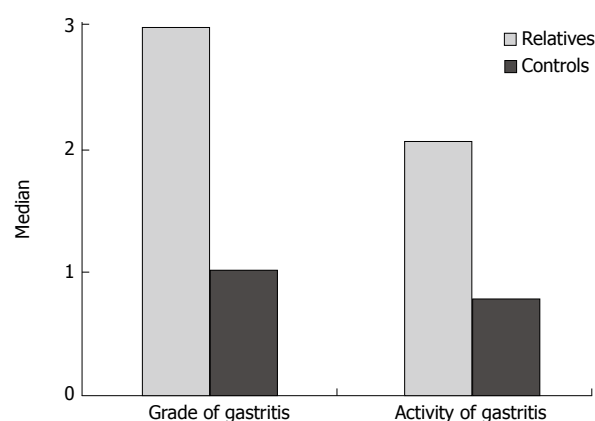
In a next step, our group compared the grade and activity of *H. pylori* gastritis in the antrum of 215 patients with gastric carcinoma or patients suffering from duodenal ulcer, showing that there are no differences in the grade and activity of antrum mucosa between the two patient groups, but significant differences in the corpus mucosa. The activity and grade of gastritis in the gastric corpus are significantly higher than those in the antrum of patients with gastric carcinoma, but only in a few patients with duodenal ulcer (Figure 2)^[16]. An additional study showed that intestinal metaplasia occurs significantly more often both in antrum and in corpus of patients with gastric carcinoma^[17]. According to Hattori *et al.*^[18], intestinal metaplasia might only be a paracancerous condition and just an expression of prior or ongoing severe inflammation, but does not cause cancer.

Gastric carcinoma risk index

In 1998, our group proposed a gastric carcinoma risk index based on previously described results. This index score consists of the presence of intestinal metaplasia (1 point) and the grade and activity of gastritis in the corpus. The maximum point is 3 when all features are present (Table 2). The positive predictive value for the presence of gastric carcinoma is 79% for score 2 and 94% for score 3^[16]. Leodolter *et al.*^[19] found that there is a strong relationship between age and the prevalence of so-called high risk gastritis with a score of 3 (Table 3).

Comparison of grade and activity in first degree relatives of gastric carcinoma patients

Due to the lack of large prospective studies, it can be argued that analyses of the topographic grading of *H. pylori*-infected gastric mucosa in patients with gastric carcinoma do not allow transfer of the findings to individuals

**Figure 2** Topographic distribution of grade and activity of *H. pylori* gastritis in individuals with early gastric cancer or duodenal ulcer (Modified from Meining *et al.*^[16]).**Figure 3** Median grade and activity of *H. pylori* gastritis in the corpus in relatives of gastric cancer patients (*n* = 237) and matched control patients (*n* = 237) (Modified from Meining *et al.*^[20]).

without gastric cancer since it might influence the status of gastric mucosa. Therefore, our group has analyzed a set of 237 first degree relatives of gastric cancer patients and 237 controls with *H. pylori* infection alone^[20]. The updated Sydney System for histological classification of gastritis^[21] consists of a four-scale semiquantitative grading system with values being not present, slight, moderate and marked.

When the risk profile is analyzed by grading the grade and activity of gastritis according to the updated Sydney System, it becomes clear that the first degree relatives of gastric cancer patients do have more severe grade and activity of gastritis (Figure 3), supporting the familial background of gastric cancer.

Gastritis status in patients with gastric adenomas and hyperplastic polyps

Adenomas of the stomach are unequivocal intraepithelial neoplasms that are limited to the gastric epithelium and can be subdivided into low-grade (adenoma) and high grade intraepithelial neoplasia (formerly dysplasia). From our routine practice, we know that the diagnosis of high grade intraepithelial neoplasia is very rare, and that most

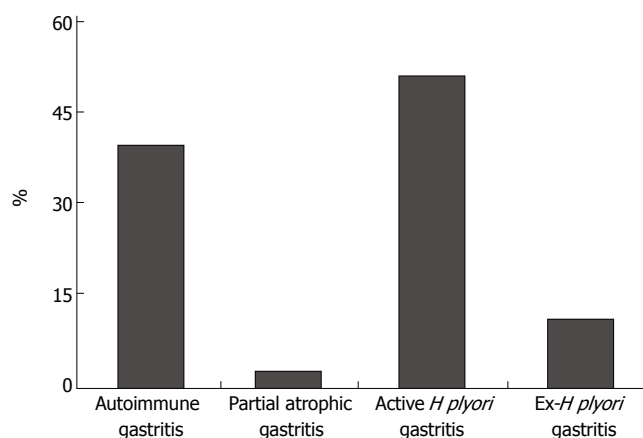


Figure 4 Prevalence of different gastritis in 118 individuals with gastric adenomas (Adapted from Meining *et al*^[25]).

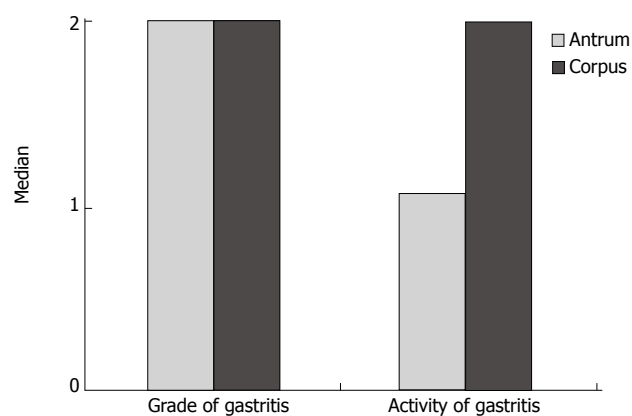


Figure 5 Grading of gastritis according to the updated Sydney System for classification of gastritis in the antrum and corpus of 91 patients with hyperplastic polyps (Adapted from Oberhuber^[27]).

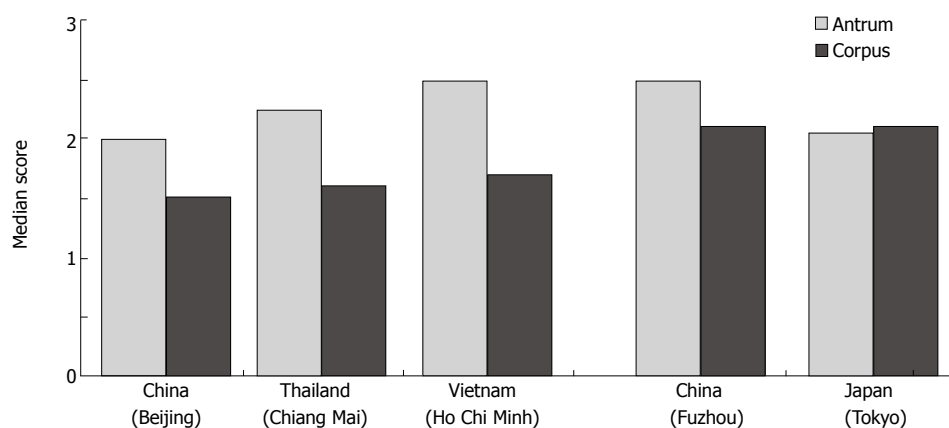


Figure 6 Mean scores of active gastritis (neutrophilic granulocytes) in the antrum and corpus in different Asian populations (Modified from Matsuhisa *et al*^[29]).

reports on follow-up studies of such lesions show that most of these “high grade lesions” are invasive cancers within a few months^[22], which probably contribute more to diagnostic uncertainty in biopsy specimens than real neoplastic progression. Adenomas can show a polypoid or a flat growth within the gastric epithelium. For gastric adenomas, the adenoma-carcinoma-sequence^[23] can also be used to represent a precancerous lesion that should be removed completely to avoid progression to cancer^[24].

In a series of 118 patients with gastric adenomas, our group showed^[25] that adenomas occur more frequently in patients with autoimmune gastritis but also in patients with *H. pylori* gastritis (Figure 4), and a subsequent analysis showed that patients with gastric adenoma have the same distribution of corpus dominant *H. pylori* gastritis like patients with early gastric cancer. In a further study, our group analyzed the gastritis status of gastric pyloric gland adenomas which is also a precancerous condition (approximately 30% show invasive carcinoma at the time of first diagnosis), and found that the proportion of atrophic autoimmune gastritis and corpus dominant *H. pylori* gastritis is high in elderly women^[26] (data not shown).

Hyperplastic polyps are also considered a precancerous lesion. A few case series in the literature and our own data^[27] indicate that hyperplastic polyps bear a risk of malignant transformation between 0.6% and 6.6%. Therefore, these polyps belong to the group of

precancerous lesions and should be removed completely. Follow-up studies showed that gastric carcinoma may develop in 8.6% of the patients at other gastric sites^[28]. As adenomas, hyperplastic polyps arise quite often in autoimmune gastritis. *H. pylori* infection is present in 37% of the cases of hyperplastic polyps. Most of these cases also show corpus-dominant *H. pylori* gastritis (data not shown) (Figure 5).

Gastritis status in populations with different risks of developing gastric carcinoma

A high incidence of gastric carcinoma is present in Fuzhou in China and in Japan, compared to Beijing in China, Thailand and Vietnam. Corpus dominant gastritis is present in Japan and China (Fuzhou), especially in the elderly whereas antrum predominant gastritis is present in Beijing (China), Thailand and Vietnam. These results correlate with the low incidence of gastric adenocarcinoma in Thai, Vietnamese and certain Chinese populations^[29] (Figure 6). Interestingly, the activity of corpus gastritis increases with age in Japanese individuals while antrum predominant *H. pylori* gastritis does not shift to more severe corpus gastritis in Vietnamese, Thai and certain Chinese populations. This is followed by a more severe colonization of *H. pylori* in cases of corpus dominant *H. pylori* gastritis. In contrast to the literature, we could not confirm the finding of improved antrum gastritis after proton-pump inhibitor

(PPI) therapy. We have found aggravated corpus gastritis as described in the literature but decreased colonization in the antrum and corpus^[30]. This might explain why increased incidence of gastric carcinoma is not reported among the millions of individuals who use acid-suppressive therapy, although corpus dominant *H pylori* gastritis is treated with PPI. Future prospective studies especially in China with its large background of individual genetic differences and environmental conditions are necessary to investigate the topography of gastritis in different Chinese populations searching for explanations for different incidences of gastric cancer in China as reported in the literature^[31]. It has been speculated that differences in environmental factors might provide a crucial clue to all these questions rather than genetic backgrounds^[32].

DISCUSSION

Corpus dominant *H pylori* gastritis is found more frequently in patients with advanced gastric carcinomas, or early gastric carcinomas and even in first degree relatives of patients with gastric cancer than in patients with duodenal ulcer or functional dyspepsia. While more than 90% of patients with gastric carcinoma are *H pylori* positive. Combined autoimmune gastritis and gastric cancer is very rarely found. On the other hand, 30% of patients with hyperplastic polyps and gastric adenoma of the intestinal or gastric type (pyloric gland adenoma) suffer from autoimmune gastritis^[16,26]. Corpus dominant *H pylori* gastritis is mainly diagnosed in cases infected with *H pylori*. All the above studies have confirmed that corpus dominant *H pylori* gastritis bears a higher risk developing gastric neoplasms rather than other forms of *H pylori* gastritis.

It is difficult to explain the different incidences of gastric cancer in patients with corpus dominant *H pylori* gastritis and autoimmune gastritis, because at least 50% of autoimmune gastritis are probably induced by *H pylori*^[2], indicating that more than one risk exists in patients with precancerous lesions and patients with corpus dominant *H pylori* gastritis have a higher risk than those with autoimmune gastritis. Corpus dominant *H pylori* gastritis is a risk factor for gastric carcinoma in individuals with precancerous conditions and in the first degree relatives of gastric carcinoma patients^[20,25]. The predictive value of corpus dominant *H pylori* gastritis needs to be investigated in future prospective studies.

Lee *et al.*^[33] are the first to consider the possibility of differences in acid secreting capacity in answering the underlying causes of corpus dominant *H pylori* gastritis. According to their hypothesis that low acid output leads to more severe gastritis, which has been confirmed later^[34,35]. Moreover, it is important to emphasize that the proposed gastritis-atrophy-intestinal metaplasia- dysplasia-carcinoma sequence by Correa^[1] is an ideal model only and fails to be proven in most cases^[18]. But intestinal metaplasia (as a paracancerous phenomenon) is frequently picked up in biopsy specimens from individuals with corpus dominant *H pylori* gastritis, indicating a close relationship.

Whether *H pylori* eradication is capable of preventing gastric cancer is to be clarified in prospective studies such as the German PRISMA study^[36]. Eradication stops at least

the progression of intestinal metaplasia and atrophy^[37] and improves gastritis^[38]. However, it has been indicated that “a point of no return” might have been reached before *H pylori* eradication cures the infection. This possibility is demonstrated by the development of 3 early gastric carcinomas in 92 patients 4 and 5 years after *H pylori* eradication and complete remission of MALT lymphomas in our German MALT lymphoma study^[39] and 3 additional cases having a similar clinical history (Malfertheiner’s personal communication). Further proof is given by the study of Wong *et al.*^[40] who showed that the incidence of gastric carcinoma is similar in individuals receiving eradication therapy or placebo during a 7.5-year follow-up period. It was reported that if no precancerous lesions (atrophy/dysplasia/intestinal metaplasia) are present, eradication of *H pylori* leads to no gastric cancer^[40-42]. Another factor that should not be disregarded is a genetic background shown by the analysis of first-degree relatives of individuals with gastric carcinoma^[43-46]. Whether metaplasia and atrophy are precancerous or paracancerous lesions^[18], they may not have a great clinical impact since these lesions occur especially in individuals with an elevated risk of gastric cancer^[47]. Large prospective studies are needed to further analyze the risk factors for gastric cancer and to clarify whether and when eradication therapy is capable of preventing gastric carcinoma.

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Indications for 5-aminosalicylate in inflammatory bowel disease: Is the body of evidence complete?

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Abstract

Mesalazine is a safe drug, although adverse events may be seen in a minority of patients. This applies also to pregnant women and children. The role of mesalazine in combination therapy to improve efficacy and concomitant drug pharmacokinetics, or in chemoprevention against inflammatory bowel disease (IBD)-related colonic carcinoma has not yet been completely elucidated. Therapeutic success of mesalazine may be optimized by a combination of high dose and low frequency of dosage to improve compliance. Therefore, due to its superior safety profile and pharmacokinetic characteristics, mesalazine is preferable to sulphasalazine. This paper reviews the literature concerning mechanisms of action, indications and off-label use, pharmacokinetic properties and formulations, therapeutic efficacy, compliance, paediatric indications, chemoprevention, and safety issues and adverse event profile of mesalazine treatment *versus* sulphasalazine. It also highlights these controversies in order to clarify the potential benefits of mesalazines in IBD therapy and evidence for its use.

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Key words: Mesalazine; Sulphasalazine; Review; Ulcerative colitis; Crohn's disease; Treatment; Chemoprevention; Pregnancy; Adverse events

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INTRODUCTION

In the late 1970s, elegant studies revealed that 5-aminosalicylate is the active moiety of sulphasalazine in patients suffering from ulcerative colitis (UC) and Crohn's disease (CD)^[1,2]. Since then, 5-aminosalicylate has become the gold standard first line therapy for patients with UC, although its use in CD remains controversial. After 25 years, discussion of monocomponent 5-aminosalicylate with regard to its efficacy in comparison with sulphasalazine^[3,4], drug profile^[5,6], precise indications^[7,8], and adverse events^[9,10] continues to be ongoing and lively. This review highlights these controversies in order to clarify the potential benefits of mesalazines in inflammatory bowel disease (IBD) therapy and evidence for its use.

CONSIDERATIONS 1 AND 2

How efficacious is mesalazine in comparison with sulphasalazine in patients with ulcerative colitis and which is the most effective mesalazine?

The therapeutic efficacy of salicylazosulphapyridine (SASP) in inducing and maintaining remission in patients with UC has been recognized for over 60 years^[11]. SASP is a conjugate of 5-aminosalicylate and sulfapyridine. 5-aminosalicylate was identified as the principal effective component of this conjugate in the 1970s^[12], and remains the starting point for the clinical use of monocomponent 5-aminosalicylate or mesalazine. Although the exact mechanism of action of mesalazine/SASP has still to be elucidated^[12,13], several potential mechanisms have been suggested, including 5-aminosalicylate-induced inhibition of inflammation by interfering with the metabolism of arachidonic acid, prevention of mucosal generation of leukotrienes and prostaglandins^[14], scavenging of free radicals^[14,15] and mechanisms only recently identified involving inhibition of nuclear factor-kappaB (NFκB) and induction of apoptosis^[16-20]. Further properties include changes in the production of immune globulins and diminished production of interleukin-1 and partial inhibition of platelet activating factor (PAF) expression, resulting in a decrease in leucocyte trafficking^[21].

5-aminosalicylate is believed to act in the damaged epithelial intestinal layer, where it is transformed into the inactive acetylated 5-aminosalicylate, which is subsequently filtered and excreted by the kidneys. As a result, the therapeutic activity and efficacy of 5-aminosalicylate are related to its intraluminal concentration^[6,13,22,23]. It has been argued that increasing dosages of oral mesalazine are

not correlated with enhanced efficacy. Although this has never been the subject of an extensive study^[3], a double-blind study in 321 patients has found no significant dose response between mesalazine (1.5 g/d, 3.0 g/d and 4.5 g/d)^[24]. Theoretically, however, dosing of mesalazine above the enzymatic (acetylating) capacity of the epithelial layer increases the subepithelial concentration of mesalazine^[25], thereby increasing its potential efficacy^[14], a finding that is corroborated by studies demonstrating a dose-response relationship of oral formulations^[26-29]. These findings have been recently corroborated by a post-hoc analysis of 423 patients out of the original 687 patients from the ASCEND I and II studies, in which daily oral use of 4.8 g mesalazine (Asacol[®]) was compared to 2.4 g mesalazine, showing that the higher dosage is superior in patients with moderately active UC^[30]. There is little doubt that registered doses of oral mesalazine are effective in treatment of active UC^[31]. Indeed, the recent (2004) British Society of Gastroenterology (BSG) guidelines^[32], recommend a combination of topical and oral mesalazine as first line therapy in the treatment of distal mild to moderate UC.

More contentiously, recent Cochrane meta-analyses of the capacity of mesalazine to induce or to maintain remission^[3] in UC patients are not in favour of mesalazine over SASP, particularly in maintaining remission. However, SASP is not as well tolerated as 5-aminosalicylate^[3]. Therefore, these authors question whether there is a clinical advantage of monocomponent preparations. However, it should be noted that mesalazine and SASP are equally effective in the 12-mo trials. It is only the 6-mo data that differ. The conclusions made from this meta-analysis are further flawed due to the following reasons. Although the authors have made considerable efforts to group studies with the same patients and outcome variables, these meta-analyses are based on different patient groups from various hospital settings. Moreover, meta-analyses may lead to incorrect conclusions, in particular when smaller studies are added to perform the analysis, as has been shown by the challenging and controversial findings of LeLorier *et al*^[33]. They showed that performing well-conducted meta-analyses with available data from small-scale studies leads to incorrect conclusions as exemplified by contradictory findings in properly designed and statistically powered prospective trials, performed to verify the conclusions from these prior meta-analyses. In addition, there is a lack of trials using high-dose mesalazine, with intention-to-treat analysis, and including registered compliance rates. Notwithstanding these limitations, and others posed by the authors (such as lack of standard UC-index of severity and different, partly obscured, treatment strategies), it could be concluded that these Cochrane-analyses showed that efficacy of oral SASP or mesalazine in induction and maintenance treatment in UC is more or less equivalent^[3,4]. Therefore, a final decision as to the choice of therapy depends largely on other factors such as adverse events and drug profile, patient's preference, and costs. Knowledge of drug profile and pharmacokinetic characteristics may be helpful in determining the correct choice in oral therapy for UC, although all compounds have proven their efficacy in colonic disease^[31,34]. In the

early trials, somewhat surprisingly, oral balsalazide therapy was shown to be superior over mesalazine^[35,36], in addition to more expected results such as better tolerability than SASP^[37,38]. However, it has since been contested that the interpretation and clinical implications of these findings are ambiguous^[34,39-41]. Direct comparative studies with well-defined clinical presentations (pancolitis versus left-sided colitis), and well-defined consistent primary study goals, such as number of patients in remission after 8 or 12 wk, are necessary to prove the therapeutic superiority primarily and secondarily, to justify preferences^[31,40].

Another important issue relates to the use of topical therapy. Where possible, topical therapy, either as an enema or a suppository, can be an excellent alternative to oral therapy, both for active^[42-44] and quiescent disease^[45,46]. To adequately cover the extent of the diseased distal colon, enema volume has to be varied^[47], whereas all rectal dosages above 1 gram seem equipotent^[48]. In addition, new entities such as gels and foams may be a patient friendly alternative^[49-51]. Furthermore, regarding combination therapy, it has recently been reported that the combination of oral (4 g/d) and rectal (1 g/d) mesalazine therapy significantly improves remission and improvement rates in extensive mild to moderate active UC^[52]. Combination therapy may, in addition, protect against progressive extension of distal disease^[53].

The ideal dosage for maintenance treatment of UC has never been studied, and most studies included in the most recent Cochrane analysis have used relatively low dosages of mesalazine^[4]. The authors concur that the ability to make general conclusions is limited due to the lack of standard indices, and treatment specifications. A dose-response rate could not be found, probably due to the small number of patients included in this subanalysis. However, it should be noted that the use of olsalazine (Dipentum[®]) may be limited owing to its diarrhoea-inducing capacity^[4]. Nowadays many clinicians believe that the same dose of mesalazine required to induce remission should be continued as maintenance therapy, particularly if higher doses are required to induce remission^[34].

CONSIDERATIONS 3 AND 4

Is mesalazine effective in Crohn's disease and is the pharmacokinetic (mesalazine release) profile of importance?

The use of 5-aminosalicylates in patients with CD is more controversial than its use in UC. The landmark study of Singleton *et al* in 1993^[54], has demonstrated a dose-response relationship with high dose (4 g/d) mesalazine. However, mesalazine therapy appears clinically beneficial only in specific subgroups, such as patients who have recently undergone ileocecal resection to prevent relapses^[55,56]. The efficacy of mesalazine in the overall heterozygous CD population is inconsistent, both in literature and in practice. Several authors advocate the use of mesalazines in mildly active CD^[8,57], although a more critical approach, namely mesalazine is not efficacious in CD, is equally contested by others^[7,58]. One meta-analysis, including data from unpublished studies, has shown a significant effect of mesalazine (4 g/d) compared to placebo in reducing CDAI

score 63 *vs* 45 (ITT) ($P = 0.04$) and 83 *vs* 57 per protocol, ($P = 0.02$)^[8]. The clinical relevance of these differences has been questioned^[58]. Nevertheless, in early studies of mesalazine use in CD, beneficial effects have been observed, which could not be reproduced in larger studies in referral centres^[8,54,55,59,60].

The therapeutic effects of mesalazine (1 g t.i.d), 6-mercaptopurine (50 mg/d), and placebo were studied for 2 years following surgery, which revealed that the relapse rate in the 6-mercaptopurine group was 50% compared with 58% and 77% in the mesalazine and placebo groups, respectively, showing a statistical significance only for 6-mercaptopurine^[61]. In another open randomized study, comparing properly dosed azathioprine and mesalazine (1 g t.i.d), no statistically significant difference was observed in the cumulative risk of clinical relapse between the two treatment regimens ($P = 0.2$), which may be due to a type 2 error^[62]. A potential role of mesalazine in maintenance therapy of CD following medically induced remission is questioned^[63].

Others believe that only SASP and not mesalazine, may be indicated for but strictly limited to mildly active colonic CD^[7]. This may reflect the colonic release profile of SASP, which clearly differs from the release profile of mesalazine delivered in microgranules, which has been shown to reduce postoperative recurrence in CD patients with small bowel involvement only (mesalazine 22% *vs* placebo 40%, $P = 0.002$)^[56,64]. Localization of the disease differs considerably between patients. This clinical heterogeneity may lead to differences in efficacy between mesalazine formulations. Drug release profiles of mesalazine moieties noticeably vary^[6]. Mesalazine incorporated in ethylcellulose microgranules (Pentasa®) releases mesalazine from the duodenum through the colon in a gradual manner at all pH levels^[6,22,65]. Conversely, mesalazine with a Eudragit-L-coating (Salofalk®) releases mesalazine exposed to a pH level of 6 to 6.5, limiting its action to the mid small intestine and onwards, whereas a similar formulation with Eudragit-S-coating (Asacol®), releases when exposed to pH levels of 7, corresponding approximately to the last part of the small intestine. The prodrugs sulphasalazine (Azulfidine®), balsalazide (Colazal®) and olsalazine (Dipentum®) release 5-aminosalicylate in the azo-reductase containing bacteria-rich colon. Thus, there exist formulations which release 5-aminosalicylates in specific regions of the intestine, while others continuously release 5-aminosalicylates in both the small and large intestine^[6,65]. Regionalization of mesalazine delivery is further influenced by enteral motility and anatomy, as exemplified by various recurrence rates after mesalazine use in Crohn's disease patients with different anastomotic configurations following resection^[66]. The pharmacokinetics of various mesalazine moieties has recently been reviewed more extensively^[5,67].

Since intraluminal concentrations of mesalazine appears to determine therapeutic efficacy, regional targeting of mesalazine is considered to be important^[6,60,64]. Remarkably, several generic oral formulations of mesalazine-containing preparations have been approved based on bibliographic files, without conductance of thorough bio-equivalence studies^[6]. Clearly, generic preparations with undocumented release profiles cannot be compared with well-documented

formulations^[5,6]. Nevertheless, "it is as yet unclear whether any specific formulation has shown site specificity for Crohn's disease"^[8], and this pharmacokinetic issue has yet to be the subject of a clinical trial. Aside from these pharmacokinetic considerations, the potential of mesalazine in CD has yet to be fully determined although the recent BSG guidelines stated that high-dose (4 g/d) mesalazines could be used as an initial therapy for mild-ileocolonic CD^[32]. However, a critical approach is warranted, as concluded by the authors of a Cochrane review concerning any use of 5-aminosalicylates in Crohn's disease patients^[63].

CONSIDERATION 5

Does non-compliance contribute to insufficient efficacy of mesalazine in maintenance therapy?

Another often overlooked issue in therapeutic efficacy is lack of compliance with therapeutic regimens. This problem has been recognized as a pitfall in conducting IBD maintenance therapy trials as early as 1982^[68]. Although reported compliance in clinical trials is usually in excess of 90%, such high levels of compliance are not necessarily continued in everyday practice. Indeed, a high rate of non-compliance up to 50%, has been reported in IBD-patients^[68-70] with a significant impact on treatment outcome which in UC at least is the most important predictor for relapse^[71]. Although medication compliance is generally good in acute disease, compliance rates in maintenance therapy decrease considerably once remission is achieved. The interpretation of maintenance studies without measurements of drug levels may therefore be difficult^[68]. One of the key risk factors for non-compliance is the number of pills and multiple medications, a risk factor that is common in IBD patients, and in particular in those taking high dosages of mesalazine in multiple dosing regimens^[70]. Reduction of the number of dosages, preferably with patient friendly formulations such as granules, without decreasing the dosage would be expected to improve the outcome of mesalazine therapy, particularly during maintenance therapy^[72].

CONSIDERATION 6

What are the safety considerations for mesalazine versus SASP?

The short-term use of mesalazine is perceived to be relatively safe^[73]. However, specifically olsalazine has been shown to induce diarrhoea in up to 17% of treated patients, probably induced by the release-profile modifying azo-bond which is also used in balsalazide and SASP, the latter two showing similar prosecretory effects in elegant *in vitro* studies^[74]. To estimate the more severe potential risks of mesalazine, most data are obtained from case reports concerning adverse events that have been published, especially relating to renal damage^[75-79] and pancreatitis^[76,80-84], both are idiosyncratic phenomena^[85]. Interestingly, 4-ASA enemas may be used when 5-ASA-induced pancreatitis occurs^[86]. Predictably, SASP containing 5-aminosalicylate, has also been associated with pancreatitis^[83,87], nephrotic syndrome^[88,89], and many other

detrimental events. The latter is reportedly associated with the sulfa component of SASP^[90,91]. In a recent report on the usage of SASP and mesalazine for ≥ 5 years in nearly 700 patients, side effects are reported most frequent in SASP-treated patients (20% *vs* 6.5%)^[92]. Evaluation of the number of prescriptions *versus* the number of central adverse events has ascribed superior safety to SASP^[9]. The methodology of this observation remains controversial^[93-95]. For short term therapy, mesalazine is widely perceived to be better tolerated than SASP, as has been measured by several methods^[73,92]. Moreover, mesalazine is a good alternative when SASP has to be withdrawn due to adverse events^[96]. In addition, other adverse events occur less commonly, or are less reported, a known flaw in epidemiological data obtained from spontaneous reporting. Therefore, many of the well-known adverse events ascribed to SASP are expected to be underreported^[95]. Overall, mesalazine is a safe drug, but its use bears a small risk for idiosyncratic renal damage and pancreatitis^[10,97-99].

CONSIDERATION 7

Should off-label use of mesalazine be considered?

Fertility, pregnancy and nursery: Conception and pregnancy are common events in the cohort of IBD patients, owing to the fact that many patients with IBD are of child-bearing age without diminished fertility, although SASP and anecdotically mesalazine have been associated with reversible, decreased male fertility^[100,101]. Ideally, conception and pregnancy take place during periods of IBD remission, without use of medication^[100,102,103]. However, this is not always feasible. Of note is that the progression of pregnancy and foetal development are more endangered by active, insufficiently treated IBD, than by the majority of pharmacological agents^[102,104]. Many drugs including mesalazine, that are used for IBD may cross the placenta^[65,105]. Therefore, toxicity and teratogenicity cannot be ruled out completely. However, the safety of mesalazine is very well documented^[103,105-110], although the safety of oral dosages above 3 g mesalazine per day during gestation has not been documented^[105]. In addition, concerns about the rate of stillbirth remain, although this may be due to activity of the disease^[110]. One incidence of renal affection in utero in a foetus where the mother received 4 g mesalazine/day has been reported^[111]. In addition, the topical use of mesalazine in enemas appears to be safe^[112]. In contrast, SASP is associated with several congenital abnormalities. Although causality has not been proven, the concomitant use of folic acid is recommended^[113-115]. Pregnancy outcome in patients using mesalazine is equivalent to that in the non-IBD population^[102,103,105,109].

Lactation is not considered to be a contraindication for mesalazine use, although minor concentrations of mesalazine have been detected in breast milk^[116]. One case of diarrhoea in a breast-fed child of a 5-aminosalicylate treated mother has been reported^[117] and cerebral thrombosis has been recently reported in the child of a breast-feeding mother on mesalazine therapy^[118]. However, it is uncertain whether mesalazine contributes to the latter

condition.

Paediatric use of mesalazine: The incidence of IBD in children appears to rise in Eastern and Western countries^[119-122]. However, not all authors agree^[123]. Although the medical approach to IBD in children is largely similar to that of adults, its treatment aims differ from the adult population^[124,125]. Mesalazine may be used in mild to moderate or severe UC, alone or in combination with steroids^[125] and dosages in children above 12 years of age are similar to those of adults. Therapy for younger children is usually 20-50 mg mesalazine per kilogram bodyweight given in two to three separate dosages per day^[126]. The pharmacokinetic profile of mesalazine pellets is comparable to that for adults in these young patients^[127]. Maintenance therapy is habitually half that dose with a minimum of 750 mg mesalazine daily. The incidence of adverse events in children is similar to that observed in adults, although rare observations such as IBD-mimicking^[128] and pericarditis^[129] have been reported. In a paediatric study comparing SASP with mesalazine, the majority of patients prefer mesalazine, due to its superior properties regarding ease and frequency of administration and its better safety profile^[130]. The problems regarding acceptance of the disease and its treatment in this patient group supports mesalazine use over SASP^[130,131]. In contrast to treatment in adults, paediatricians tend to use mesalazine in non-stenotizing CD and if necessary, concomitant enteral nutritional therapy can be used in active disease or as a maintenance therapy^[127,132]. However, none of these indications has been subjected to large controlled trials in children.

Chemoprevention of IBD-related colonic cancer: Traditionally UC and more recently CD have been associated with enteral adenocarcinoma, although the risk appears to be limited to patients with chronic inflammation^[133,134]. Interestingly, several studies and reviews refer to potential chemoprotective properties of mesalazine moieties in prevention of this type of cancer^[18,19,135-139], similar to the alleged chemoprotective properties of acetylsalicylic acid, although the latter remains contraindicated in patients with IBD. Laboratory and other findings support the hypothesis of mesalazine as a chemoprotective agent^[138]. These chemopreventive properties include selectively inducing apoptosis in colorectal cancer (CRC) cells and stabilising effects on micro-satellites^[19]. In addition, the incidence of CRC has been demonstrated to be reduced in a case-control study (odds ratio 0.19, 95% confidence interval: 0.06%-0.61%) if patients use mesalazine doses above 1.2 g daily^[137]. Nevertheless, it could be argued that the decreasing relative risk for development of colonic carcinoma in patients with IBD is related to successful therapy of chronic active disease *per se*, and is thus unrelated to mesalazine therapy^[27,138]. In short, prospective and comparative data are lacking, but the indication for mesalazine as a chemopreventive agent looks promising, as recently concluded in a meta-analysis^[140].

CONSIDERATION 8

Does mesalazine have a role in combination therapy?

Concomitant use of mesalazine formulations may be beneficial, as its efficacy seems related to intraluminal

concentrations. Combination therapy of oral and rectal mesalazine has been investigated. Interestingly, higher mucosal concentrations of mesalazine^[27] have been associated with the decreased relapse rates but not with higher remission rates^[141]. Prior studies have reported that earlier remission can be achieved using combination therapy in left-sided UC^[142], and that a longer duration of remission has been observed when enemas are combined with low oral dosages of mesalazine^[143]. In addition, recent studies have also demonstrated the benefits of combining high-dose oral mesalazine with topical therapy in terms of remission and improvement rates in patients with UC^[52], and protection against increase of disease extent^[53]. Again, extensive well-designed dose-response trials are lacking and no final conclusion can be drawn from these sparse data.

Another interesting approach to the treatment of UC patients may be dual therapy with a combination of mesalazine and another inflammation-modifying drug such as butyrate^[144,145], fraxiparin^[146], or allopurinol^[147]. Also, preliminary studies investigating combination therapy with mesalazine and probiotics support their use^[148]. It should be noted, however, that as these reports are open-label studies, conclusions may not be as valid as those obtained from studies using classical approaches such as increasing mesalazine dosage or switching of type of drugs. Although the combination of mesalazine and steroids may, theoretically, have synergistic effects, this concept has yet to be substantiated by clinical trials, although for topical therapy, the combination of beclomethasone and mesalazine has been proved beneficial in a small open-label study of patients with mesalazine-refractory ulcerative proctitis^[149]. The combination of azathioprine (a potent immunosuppressive drug) with mesalazine (a milder agent) seems counter-intuitive. Proper studies are lacking once again, although one study has suggested that mesalazine may interfere with azathioprine metabolism, inducing a higher concentration of supposedly active metabolites, such as 6-thioguaninenucleotides^[150]. After 25 years of mesalazine use, it is clear that the value of combination therapy, whether beneficial or detrimental, remains to be fully elucidated.

SUMMARY

Decisions regarding treatment options for IBD must be carefully weighed following a careful benefit-risk analysis. The immediate goal of controlling active diseases must be balanced against the long-term goal of keeping patients asymptomatic on a therapy with acceptable toxicity. Large prospective and retrospective cohorts demonstrate that mesalazine is a safe drug, although adverse events may be seen in a minority of patients. Overall, in UC, mesalazine is beneficial in mild to moderate active diseases^[31,151-153]. Maintenance therapy with mesalazine is also well documented^[151], although it remains contentious as to which dosage and dosing frequency are optimal. With respect to a high compliance and good therapeutic success, the combination of high dose and low frequency is ideal and mesalazine preparations which can provide this are optimal. Indeed, preliminary studies support this type of therapeutic regimen although further studies are

needed to confirm these findings^[154,155]. Topical therapy is safe and effective and can be used to reduce systemic concentrations of mesalazine or its methylated metabolite, particularly in those prone to develop adverse events^[43]. The alleged potential of mesalazine therapy in CD requires further clarification, but the use of mesalazine delivered in microgranules postoperatively including small bowel CD only, and SASP in colonic disease, is scientifically corroborated^[56,58,61]. The limited data available support the use of the low toxicity mesalazine agents in children. The use of mesalazine in pregnancy and during breast-feeding, indicates that mesalazine is very likely to be safe. Preliminary studies suggest that 5-aminosalicylate also plays a role as a chemoprotective agent in reducing the risk of developing colorectal cancer, although further studies are needed to confirm this effect. The combination of various inflammation-modifying drugs with mesalazine is a sparsely investigated field, but initial data are encouraging.

Overall, mesalazine is a safe and effective drug with a pivotal role in UC patients and a limited role in CD patients. Although clinical superiority in comparison with SASP has yet to be proven, the superior safety profile and pharmacokinetic characteristics of mesalazines definitely advocate their use as the treatment of choice when treatment with 5-ASAs is indicated.

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REVIEW

Management of pseudomyxoma peritonei

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Abstract

Pseudomyxoma peritonei (PMP) is a rare disease. It refers to a progressive disease process within the peritoneum which originates from the appendix or ovaries and is characterised by the production of copious amounts of mucinous fluid resulting in a "jelly belly". If untreated the condition is fatal. The traditional approach to PMP is based on repeated surgical debulking procedures, often associated with intraperitoneal or systemic chemotherapy. The natural history of this disease has been drastically modified since the introduction of a new surgical approach defined as a peritonectomy procedure. This paper is to review the literature on this treatment strategy.

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Key words: Pseudomyxoma peritonei; Peritonectomy; Hyperthermic intraperitoneal chemotherapy; Sugarbaker procedures

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INTRODUCTION

Pseudomyxoma peritonei (PMP) is a rare disease^[1]. It refers to a progressive disease process within the peritoneum which originates from the appendix or ovaries and is characterised by the production of copious amounts of mucinous fluid resulting in a "jelly belly". If untreated the condition is fatal. With the progress of immunohistochemistry, PMP should be considered as a border line malignant disease because of its inevitable persistence and progression. PMP is an indolent disease, which is most prevalent in women aged between 50 and

70 years. The cases of ovarian origin outnumber those of appendiceal origin. A large proportion of ovarian tumours are secondary to appendiceal tumours. More women appear to suffer from this condition than men. However, the appendix is the primary source of most PMPs, which may then spread to other sites like the ovaries^[2]. Clinically, although painless, deterioration of general health begins long before diagnosis. Acute presentation during advanced stage of the disease is common. The main complaints are abdominal pain and distension besides a host of non-specific symptoms. Inflammatory changes associated with peritoneal tumour implants can lead to fistula formation and adhesions, which in turn can cause intermittent or chronic partial bowel obstruction. Localized masses are frequently present in PMP of appendiceal origin. Surprisingly, signs and symptoms of cancer such as cachexia are rare. The characteristic PMP dissemination within the peritoneal cavity is defined by Sugarbaker^[3] as a complete redistribution phenomenon, indicating a complete and sequential invasion of the peritoneal cavity with large tumor volume localization at predetermined anatomical sites and minimal invasion at other sites. The modalities of dissemination are strongly influenced by the histopathology of the primary tumor^[4,5]. The traditional approach to PMP is based on repeated surgical debulking procedures, often associated with intraperitoneal or systemic chemotherapy. The natural history of this disease has been drastically modified since the introduction of a new surgical approach, proposed by Sugarbaker^[3] who defines it as a peritonectomy procedure consisting of the complete removal of the tumor. Surgery is followed by local drug administration aimed at eliminating microscopic and/or minimal residual disease left in the abdominal cavity following surgical manipulations^[6]. The additional effects of hyperthermia, through the use of a special pump, increase local tissue drug concentration and consequently antitublastic drug activity^[7]. This technique has been defined as hyperthermic intraperitoneal chemotherapy (HIPEC). These methods can achieve satisfactory results in patients with PMP^[8-12]. We here review the application of peritonectomy and hyperthermic intraperitoneal chemotherapy in the treatment of PMP.

PERITONECTOMY PROCEDURE

This fundamental technique requires the removal and stripping of all tumour tissues involving the parietal and visceral peritoneum. Small cancer deposits on the visceral peritoneum, especially on the surface of tubular structures, are individually electroevaporated. Large tumour nodules

in the small bowel must be resected and all visible tumors must be removed to maximize the benefits of peri-operative intraperitoneal chemotherapy. The surgical technique has been described in detail previously and is based on the Sugarbaker principles of peritonectomy^[13,14] with a few modifications. In brief, peritonectomy procedures are performed on the basis of disease extension by the following steps: (1) greater omentectomy and right parietal peritonectomy with or without right colon resection, (2) pelvic peritonectomy with or without sigmoid colon resection as well as hysterectomy and bilateral salpingo-oophorectomy, (3) lesser omentectomy and dissection of the duodenal-hepatic ligament with or without antrectomy and cholecystectomy, (4) right upper quadrant peritonectomy and glissonian capsule resection, (5) left upper quadrant peritonectomy and left parietal peritonectomy with or without splenectomy, and (6) other intestinal resection and/or abdominal mass resection.

HIPEC

Intraperitoneal chemotherapy is only required to eradicate microscopic residual disease for its complete success. The pharmacokinetic advantage of the intraperitoneal route of drug administration is not compromised when used as a planned part of a surgical procedure. The high molecular weight of chemotherapy agents and their water solubility (hydrophilic) cause a prolonged retention in the peritoneal space. Also, use of selected drugs under hyperthermic conditions can increase cytotoxicity on the peritoneal surface but not systemic (bone marrow) toxicity. Hyperthermia can improve drug penetration into tumor tissue and optimize the dose intensity of chemotherapy on the abdominal and pelvic surfaces. The combined use of hyperthermia and intraperitoneal chemotherapy enhances the cytotoxicity of chemotherapeutic agents and increases tissue penetration by chemotherapy in cancerous tissue as compared to normal tissue. HIPEC is performed after completion of the anastomosis. The closed-abdomen technique is used for all patients. Two inflow catheters are inserted, one in the right subphrenic space, the other deep in the pelvic cavity. Two outflow catheters are inserted, one in the left subphrenic space, the other more superficially in the pelvic cavity. Six thermocouples are used to continuously monitor the inflow, outflow, and intraperitoneal cavity temperatures. Temporary abdominal skin closure is carried out following a tight continuous nylon stitch. The catheters are then connected to an extracorporeal perfusion circuit. The intraperitoneal temperature is maintained at 42.5°C during the perfusion. Different chemotherapeutic agents are used depending on the tumor histological characteristics. Intraperitoneal chemotherapy regimens are as follows: (1) cisplatin (CDDP; 25 mg/m² per liter) and mitomycin C (MMC; 3.3 mg/m² per liter)^[15] for pseudomyxoma peritonei and colorectal and gastric carcinomatosis, (2) CDDP (43 mg/L of perfusate) and doxorubicin (15.25 mg/L of perfusate)^[16,17] for mesothelioma, ovarian carcinomatosis, and sarcomatosis. The perfusate is then instilled into the peritoneal cavity at a mean flow rate of 600 mL/min.

CURRENT STRATEGY FOR COMBINED TREATMENT

Peritonectomy in combination with hyperthermic intraperitoneal chemotherapy is divided into four major steps: electrosurgery for tumour resection and peritonectomy, hyperthermic intraperitoneal chemotherapy, reconstruction, and early post-operative intraperitoneal chemotherapy. Dedicated instruments and specific surgical techniques are essential to achieve optimum results^[18-22].

Intra-abdominal dissection is facilitated by electroevaporative surgery using a 0.3 cm ball-tipped diathermy. The electrosurgical generator is set at a very high voltage between 200 MW and 250 MW. A maximal pure cut that evaporates the tissues on contact is used for dissection, which minimizes blood loss from small vessels up to 1.5 mm in diameter. Larger vessels are electrocoagulated or ligated in continuity and divided. Heat damage can be reduced by a frequent intermittent saline irrigation at the site of dissection. Heat necrosis at the tumour's margin of resection could reduce the likelihood of cancer dissemination and local recurrence.

The selection of agents for peri-operative intraperitoneal chemotherapy is based on the drug's ability to produce a cytotoxic effect over a short time period and to show heat synergy. Mitomycin C, doxorubicin and cisplatin have a slow clearance from the peritoneal cavity. The effects of these agents are potentiated by hyperthermia to achieve a maximum cancer cell kill. Intraoperative intraperitoneal chemotherapy can absorb 75%-90% of mitomycin C and cisplatin within the first hour. Despite the greatly enhanced drug cytotoxicity because of high concentrations and heat synergy, the technique is effective only in treating small volume peritoneal disease. The use of early post-operative intraperitoneal chemotherapy may be restricted due to the co-morbidity state of patients.

EFFECTIVENESS OF PERITONECTOMY AND HIPEC

Several prospective studies have shown promising results when peritonectomy and HIPEC were used to treat peritoneal carcinomatosis^[23-26]. Verwaal *et al.*^[27] demonstrated that this new treatment strategy can prolong the survival time of patients with carcinomatosis of colorectal origin. The institution that has a program to treat peritoneal carcinomatosis requires not only highly specialized human resources, but also complex technological facilities to perform the peritonectomy plus HIPEC^[28], which minimizes treatment-related morbidity and mortality and maximizes results in terms of survival and quality of life. In this context, identification of risk factors for postoperative complications is of major concern. Small-bowel perforations and anastomotic leaks are the most common complications associated with a surgical procedure unusually involving a long operative time, numerous and complex resections, peritoneal stripping, and a heated intraperitoneal chemotherapy component. Bryant *et al.*^[29] showed that patients with PMP may benefit

from the Sugarbaker procedure and have an estimated 5-year and 10-year survival of approximately 50% and 18%, respectively. In contrast, the 2-year, 3-year and 10-year survival rates of patients after HIPEC are 90%, 60%-90%, and 60%-68%, respectively. The percentage of patients with no evidence of disease at the end of follow-up after the Sugarbaker procedure ranges from 41% to 82%. Similarly, the percentage of patients alive with disease at the end of follow-up after HIPEC ranges from 9% to 35%. Mortality of the disease ranges from 2% to 31% after the Sugarbaker procedure.

DISCUSSION

Pseudomyxoma peritonei is a rare disease which is characterized by a large amount of mucinous ascites with peritoneal and omental implants. A clear understanding of its natural history has been hampered by the fact that tumors of various sites under the heading of PMP have significantly different biologic behaviours leading to dilemma in diagnosis and management. PMP is referred to an extensive mucinous accumulation within the peritoneal cavity associated with a malignant cystic neoplasm arising commonly from the appendix or ovary. Other sites include pancreas, bile duct, colon, gall bladder and urachus. No controlled clinical studies have been conducted on PMP because of its rarity (1 case/million per year), and up until now, treatment has been directed at palliation and delaying the lethal outcome that seems inevitable for these patients. Peritonectomy and hyperthermic intraperitoneal chemotherapy can prolong the survival of these patients. Peritonectomy is a complex approach and leads to postoperative complications. This technique is also employed in the treatment of other malignancies, such as gastric, ovarian, and colon cancer and peritoneal mesothelioma, in which peritoneal dissemination remains an important cause of surgical treatment failure^[30-31]. Understanding the treatment and mastery of surgical skills to manage the peritoneal surface spread of cancer can prolong the survival of selected patients. Combination of this treatment strategy with proper patient selection has reduced the mortality and morbidity. The success of peritonectomy and hyperthermic intraperitoneal chemotherapy depends on a long-term dedication to achieve the full potential of a curative outcome.

In conclusion, peritonectomy in combination with hyperthermic intraperitoneal chemotherapy is a feasible treatment for pseudomyxoma peritonei.

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COLORECTAL CANCER

Total plasma homocysteine and methylenetetrahydrofolate reductase C677T polymorphism in patients with colorectal carcinoma

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Abstract

AIM: To investigate the behaviour of total plasma homocysteine (tHcy) and its most common genetic determinant defect, the methylenetetrahydrofolate reductase C677T (C677TMTHFR) polymorphism in patients with early stage colorectal carcinoma.

METHODS: tHcy was quantified by Abbott IMx immunoassay; screening for C677TMTHFR substitution was performed by PCR and restriction analysis.

RESULTS: The frequency of the C/T and T/T genotypes of the C677TMTHFR gene polymorphism did not differ between the groups. The mean tHcy was statistically higher in cancer patients than in control subjects carrying the same C/C or C/T genotype, whereas there was no difference in the T/T homozygous carriers of the two groups. tHcy was significantly higher in the T/T homozygous carriers than in C/C and C/T genotype carriers.

CONCLUSION: The statistically significant increase of tHcy observed in C/C and C/T genotype carriers among our cancer patients is related to substrate consumption dependent on the tumor cell proliferation rate, whereas the tHcy increase observed in T/T genotype carriers of both groups probably depends on the enzymatic deficit of the homocysteine conversion to methionine and/or on the folate deficiency.

INTRODUCTION

Homocysteine, a sulfur-containing amino acid produced by the adenylation and subsequent demethylation of dietary methionine, is an essential intermediate in folate metabolism. Two enzymes and three vitamins play a key role in the regulation of circulating homocysteine levels. Of the enzymes, cystathionine- β -synthase controls the breakdown of homocysteine to cystathionine in the transsulfuration pathway, while methylene tetrahydrofolate reductase (MTHFR) is involved in the remethylation pathway, in which homocysteine is converted back to methionine. Homocysteine is present in human plasma in four forms: 1% as free thiol, 20%-30% as disulfur with itself and others thiols, 70%-80% as disulphide bound to plasma proteins. The sum of all the forms of homocysteine existing in plasma is referred as "total plasma homocysteine" (tHcy)^[1,2].

Folic acid, vitamin B₆ and vitamin B₁₂ are essential cofactors in homocysteine metabolism and a lack of them due to a deficient diet or disease can produce elevated plasma homocysteine^[2,3]. Increased homocysteine concentrations are thought to directly affect carcinogenesis by diminishing DNA methylation in critical tissues through a simultaneous increase in intracellular S-adenosylhomocysteine^[4]. Inclusion of homocysteine in the assessment of folate in carcinogenesis is important because we may deal with an issue of inadequate folate metabolism, which is indicated by the reduced function of enzymes involved in homocysteine metabolism, rather than merely a state of folate deficiency. However, to our knowledge, only one study has reported that there is no significant positive association between serum homocysteine and colorectal cancer^[5]. Despite the substantial amount of published data related to folate and colorectal carcinogenesis, the mechanisms responsible for this effect remain unclear.

Plausible mechanisms described in the literature include the role of folate in the metabolism of one-carbon units, such as methyl, methylene, and formyl groups involved in various substrates and a variety of enzymatic reactions that are intimately related to DNA and RNA synthesis and cell proliferation^[4,6]. It was recently proposed that an imbalance between biological methylation and nucleotide synthesis is a key to clarifying the mechanisms responsible for the role of folate in carcinogenesis^[4-7]. In addition, lower folate intakes, compared with higher supplements, have been shown to be associated with a significantly higher risk of *Ki-ras* mutations (prominent in colorectal neoplasia) in adenomas^[8] and carcinomas^[9].

Homocysteine metabolism has been shown to be dependent on genetic factors as well as acquired factors^[10]. Of the gene defects, the most common is the C-to-T substitution at nucleotide 677 in the coding region of the gene for MTHFR, the so-called thermolabile variant. There are an elevated homocysteine concentration and a decreased plasma folate concentration in the homozygous mutant genotype of C677TMTHFR gene. An adequate folate intake is thus required to decrease the elevated homocysteine concentration^[11,12]. The C677TMTHFR polymorphism might modulate the risk of developing neoplasia through its effects on folate metabolism. It regulates the production of thymidylate and purines for DNA synthesis and supply of methyl groups for the synthesis of methionine and DNA methylation^[4]. It has also been proposed that the protective effect of folate against the risk of colon cancer is stronger among subjects with a positive family history of colorectal cancer than among those without such a history^[13]. This study was to investigate the pattern of tHcy levels and the C677TMTHFR polymorphism in a group of neoplastic patients with non metastatic colorectal cancer, who were eligible for curative surgery, by determining tHcy levels in neoplastic patients and then clarifying whether this was due to a genetic enzymatic defect or was a consequence of cancer cell proliferation if the levels were increased.

MATERIALS AND METHODS

Subjects

Ninety-three consecutive colorectal carcinoma patients (51 men and 42 women, mean age 62 years, range 48-78 years), admitted to our surgery department for elective and curative surgery, were enrolled in the study. The criteria for exclusion from the study were as follows: malnutrition, serum creatinine level > 13 mg/L, assumption of vitamin supplements or other drugs which might interfere with plasma tHcy levels, pernicious anaemia, skin diseases, heavy smoking, and alcohol abuse. The subsite distribution of the 93 colorectal cancers showed 19 in right colon, 6 in transverse colon, 45 in left colon, and 23 in rectum. Diagnosis of adenocarcinoma was confirmed in all patients by histology and cytologic investigations. TNM stages of resected tumors were as follows: stage 1 = 6 cases (28%), stage 2 = 25 cases (27%) and stage 3 = 42 cases (45%). Histopathological grading of adenocarcinoma was: 2% well differentiated, 81% moderately differentiated, or 17% poorly differentiated (Table 1). The control group included

Table 1 Characteristics of control subjects and cancer patients

Group	Sex (M/F)	Mean age \pm SD (yr) (range)	TNM staging
Controls	100 (56/44)	64 \pm 6 (46-79)	//
Cancer patients	93 (51/42)	62 \pm 8 (48-78)	T1-3, N0-2, M0

100 healthy individuals (56 men and 44 women, mean age 64 years, range 46-79 years), enrolled with the same exclusion criteria as the cancer patients, selected from the hospital staff and people attending our out-patient surgery (Table 1). The study was approved by the local ethical committee and informed consent was obtained from all participants.

Homocysteine determination

Blood samples were collected from the antecubital vein, after overnight fasting. They were drawn into vacuum tubes containing K3-EDTA, immediately put on ice and centrifuged at 2200 r/min for 20 min at 4°C. The supernatant platelet-poor plasma was stored at -80°C until assay. Total Hcy was quantified using the fluorescence polarization immunoassay (FPIA) on the IMx analyzer from Abbott Laboratories (Abbott Park, IL, USA). The Imx Hcy assay is based on reduction of the plasma samples with dithiothreitol and subsequent conversion of free Hcy to S-adenosyl homocysteine by hydrolase in the presence of added adenosine. The sample and the tracer compete in binding to the monoclonal antibody. This reaction is followed by detection of S-adenosyl homocysteine by a fluorescence polarization immunoassay. The concentration of tHcy in plasma is inversely related to the intensity of the polarized light. The within-run CV for fasting tHcy was 2.2%-1.5% and the between-run CV was 2.2%-3.0%^[14].

Genotyping of C677T MTHFR mutation

Genomic DNA was isolated from peripheral blood leukocytes. Screening for the MTHFR 677C→T substitution was performed by polymerase chain reaction (PCR) of genomic DNA, followed by *Hinf* I digestion and agarose gel electrophoresis. The region surrounding position 677 in the MTHFR gene was amplified by PCR technique, and the alleles were identified through restriction enzyme digestion as previously described^[15]. PCR reactions of 15 μ L contained 30 ng template genomic DNA and 1.5 U AmpliTaqGold DNA polymerase and the following concentrations of reagents: 0.16 μ mol/L of each oligonucleotide primer, 1.6 mol/L of each dNTP, 15 mmol/L Tris-HCl (pH 8.0), 50 mmol/L KCl and 1.5 mmol/L MgCl₂. Verification of amplification was achieved by comparing the 173-bp products with molecular weight standards in a gel made from a combination of 3% BibcoBRL ultrapure agarose and 1.5% NuSieve GTG agarose run for 1 h at 150 V and visualized with ethidium bromide. Reaction products (5 μ L) were digested for 1 h at 37°C with 2.5 U of *Hinf* I in buffer at pH 7.9 of 10 mmol/L Tris-HCl, 10 mmol/L MgCl₂, 50 mmol/L NaCl and 1 mmol/L dithiothreitol. Genotypes were determined by analysis of restriction patterns after electrophoresis on

Table 2 tHcy plasma levels according to C677T MTHFR polymorphism [mean \pm SD (μ mol/L) (*n*)]

Group (<i>n</i>)	CC genotype	CT genotype	TT genotype
Control subjects (100)	9.01 \pm 2.76 (30)	9.71 \pm 2.34 (51)	17.56 \pm 10.81 ^{d,f} (19)
Cancer patients (93)	11.82 \pm 3.09 ^a (32)	12.45 \pm 4.46 ^b (40)	16.88 \pm 9.25 ^{d,f} (21)

^a*P* < 0.05, ^b*P* < 0.01 *vs* control; ^d*P* < 0.01 TT *vs* CC; ^f*P* < 0.01 TT *vs* CT.

Table 3 tHcy plasma levels according to CC + CT genotype of C677T MTHFR polymorphism [mean \pm SD (μ mol/L) (*n*)]

Group	(CC + CT) genotype
Control subjects	9.45 \pm 2.51 (81)
Cancer patients	12.17 \pm 3.90 ^b (72)

^b*P* < 0.002 *vs* control.

agarose gels (as above) with T at position 677, resulting in fragments of 125 and 48 bp in length and with C at position 677, resulting in a single fragment of 173 bp. We referred to the C/C genotype as wild-type, the C/T genotype as the heterozygous variant and the T/T genotype as the homozygous variant.

Statistical analysis

Normally distributed continuous variables were analysed using the Student's *t* test. To assess the normal distribution we applied the Kolmogorov-Smirnov test on each sample data. Non-normally distributed variables were analysed using the Mann-Whitney test. We used the χ^2 test for group comparison frequencies. Continuous variables were compared by Student's *t* test or the ANOVA when multiple statistical hypotheses were assumed. All *P* values were two-sided. A significance level of 95% (*P* < 0.05) was assumed. Statistical analysis was performed by SPSS (v. 11.0).

RESULTS

In the control group there were 51 heterozygous and 19 homozygous carriers of the C677T MTHFR polymorphism (Table 2), with a prevalence of 51% of the C/T genotype and 19% of the T/T genotype that corresponded to a T allele frequency of 44.5%. Among the colorectal cancer group we found 40 heterozygous and 21 homozygous carriers of the C677T MTHFR polymorphism, with a prevalence of 45.5% of the C/T genotype and 23.8% of the T/T genotype that corresponded to a T allele frequency of 46.5%. The prevalence of both the genotypes (Table 2) like the frequency of the T allele was not statistically different between the groups.

When patients were subdivided according to cancer stage (regional *versus in situ*/localized), the prevalence of T/T genotype was 21.42% (9 cases) in stage 3 and 23.5% (12 cases) in stage 1/2, while the prevalence of T/T genotype according to cancer location (colon *versus* rectum) was 21.42% (15 cases) in colon and 26.08% (6 cases) in rectum. The prevalence of T/T genotype was not statistically different when both stage and location

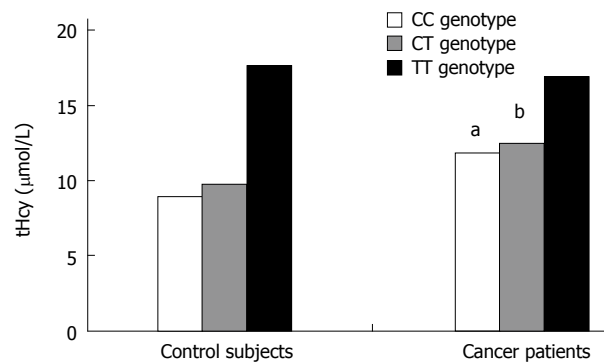


Figure 1 Mean tHcy plasma levels according to C677T MTHFR genotype, ^a*P* < 0.04, ^b*P* < 0.01 *vs* control.

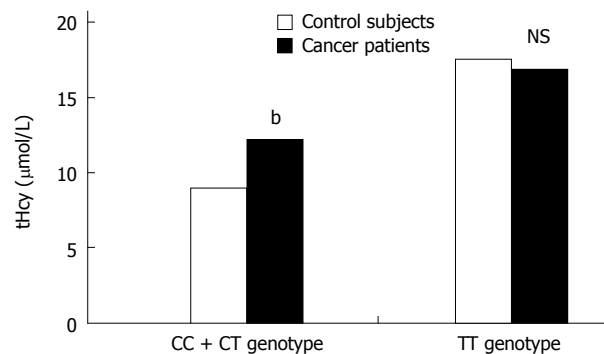


Figure 2 Mean tHcy plasma levels according to C677T MTHFR polymorphism, ^b*P* < 0.002 *vs* control; NS: Not significant.

were considered. The mean total Hcy level was statistically higher in cancer patients than in control subjects carrying the same C/C or C/T genotype (Table 2 and Figure 1, *P* < 0.04 and *P* < 0.01 respectively). In addition, when all the C/C and C/T carriers were considered together and the two study groups were compared, tHcy was still more significantly higher in the cancer group (12.17 \pm 3.90 *vs* 9.45 \pm 2.51, *P* < 0.002, Table 3, Figure 2). In contrast there was no difference in the mean total Hcy concentrations among the T/T genotype carriers of the two study groups (*P* = 0.6). In both groups tHcy was significantly higher in the T/T genotype than in the C/C and C/T genotypes, although there was no difference between the C/T and C/C genotype carriers (Table 2, Figure 2). Total plasma Hcy was not associated with tumor stage (regional *versus in situ*/localized) or location (colon *versus* rectum): 12.53 \pm 6.43 μ mol/L in colon, 13.82 \pm 6.08 μ mol/L in rectum, 12.62 \pm 5.21 μ mol/L in stage 3 and 12.97 \pm 7.15 μ mol/L in stages 1 and 2.

DISCUSSION

In our group of colorectal cancer patients the T allele frequency, like the prevalence of the C/T and T/T genotypes of the C677T MTHFR polymorphism, was similar to that in the control subjects, whereas the mean tHcy concentration was significantly increased in the cancer patients carrying the C/C or C/T genotype. This difference was even more evident when the C/C plus

C/T genotype carriers of each group were compared. This finding is difficult to explain because the increase of tHcy was not be simply due to the decreased MTHFR enzyme activity, but rather due to a more complex disorder of folate metabolism, probably partly related to the methionine-dependency of the proliferation rate of colorectal tumor cells. It is well known that most malignant cells, including colon cancer cell lines in primary histoculture need methionine, but endogenous methionine conversion from homocysteine does not meet the increased metabolic demand of these cells^[16]. Insufficient methionine synthesis in cancer cells can result from a lack of reduced folate as a cofactor in the homocysteine 5-methyl-tetrahydrofolate methyltransferase reaction. In fact the reduced folates may be drained into other pathways that are enhanced in proliferating cells, such as the synthesis of purines and pyrimidines. As a consequence there is an increase of homocysteine in dividing cells, resulting in a greater egress of it from such cells. High tHcy levels may therefore be a phenotypic expression of malignancy, as well as a marker for cancer cell activity.

Serum concentration of homocysteine increases with folate deficiency because the metabolic disposal of homocysteine depends on a remethylation reaction in which N-5-methyltetrahydrofolate serves as a co-substrate with homocysteine^[17]. In practice, an elevation of this amino acid has been found to be a more sensitive indicator of cellular folate depletion than its blood folate concentrations^[18]. The importance of homocysteine concentration as a biological marker is highlighted by Kim *et al*^[19], who found that blood folate and homocysteine are correlated with colonic mucosal folate concentration.

Since we did not measure plasma folic acid, vitamin B12 and B6 concentrations, if the increase of tHcy observed in our cancer patients is a consequence of nutritional deficiency or substrate consumption induced by active neoplastic proliferation remains unknown. Assuming that our cancer patients had the same nutritional habits as our control subjects, the hypothesis is that more active consumption of substrates is more likely secondary to the metabolism of one-carbon units related to DNA and RNA synthesis^[5-8].

According to previous reports regarding the Italian population^[20], we found a similar and increased prevalence of T/T genotype carriers, in both our cancer and control groups (21% and 22.4% respectively). The total Hcy concentration in these subgroups was similar both in cancer patients and in control subjects, suggesting that the T/T genotype has a stronger influence on tHcy concentration, thus overcoming the biochemical cancer effect.

It has been reported that the T/T genotype of the C677T MTHFR gene polymorphism is associated with a decreased risk of colorectal cancer^[21,22]. We can argue that in our subjects, both in the control and cancer groups, there is a depletion of folate. In the folate deficiency, the high activity of the enzyme or the C/C genotype may be disadvantageous because 5, 10-methylenetetrahydrofolate is converted and the thymidylate pool is depleted. Increased risk for T/T genotype *versus* C/C genotype would be seen in the folate-depleted situation if aberrant

DNA methylation is a primary mechanism, but no such increase has been observed^[23]. We have no data about serum folate, but the increase of total Hcy observed in our T/T genotype carriers may also be an index of low folate status, a condition that confers no protection against cancer risk, as reported by Ulvik *et al*^[24]. Moreover, because the number of individuals carrying the T/T genotype in both our groups is low, it is possible to hypothesize that a modest influence of cancer on tHcy exists, although it was not shown by our data. Of note is that a previous study investigating a large cohort of Caucasian colorectal cancer cases and matched controls, reported that C677T MTHFR polymorphism is associated with total Hcy^[24]. It cannot be excluded that the difference found in our study may have been biased by the relatively small number of patients and controls with different genotypes. The relatively high SD value for T/T genotype carriers supports this possibility. The T/T genotype of the C677T MTHFR variant has less than one third of the functional activity of the normal MTHFR enzyme and is associated with reduced cellular folate and methionine levels, reduced cellular ratio of S-adenosylmethionine to S-adenosylhomocysteine, as well as elevated homocysteine levels^[25]. Based on Frosst's data, a normal C/C homozygote has 100% activity, a C/T homozygote approximately 65% activity, and a T/T homozygote approximately 30% activity^[26]. Finally, it is important to emphasise that hyperhomocysteinaemia is a well-known and independent risk factor for thrombosis^[27]. Moderately elevated levels of tHcy are associated with an increased risk of thrombosis, through mechanisms that are incompletely understood. In particular, there is evidence that hyperhomocysteinemia increases the risk of venous thromboembolism approximately two to four-folds^[28-30], contributing to the hypercoagulable state that characterises the malignant disease^[31].

In summary, the results of our study suggest that the modest but significant increase of plasma tHcy observed in the C/C and C/T genotype carriers in our cancer group, may be related to the methionine-dependent proliferation rate of colorectal cancer cells and moreover, may act as a permissive factor for thrombosis in the context of cancer thrombophilia. The tHcy increase observed in T/T genotype carriers in both groups, on the other hand, is probably dependent on the enzymatic deficit associated with the homocysteine conversion to methionine and/or the depletion of folate.

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Distinct patterns of mucosal apoptosis in *H pylori*-associated gastric ulcer are associated with altered FasL and perforin cytotoxic pathways

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expressing cells were increased in *H pylori*-infection and correlated with epithelial apoptosis. Perforin-expressing cells were also increased in GU compared with *H pylori*-gastritis.

CONCLUSION: Epithelial apoptosis is increased in *H pylori*-infection and correlates to FasL- and perforin-expression by T cells. Expression of perforin is correlated with the tissue damage, and may represent the enhancement of a distinct cytotoxic pathway in GU. Increased expression of FasL not paralleled by Fas on T-cells and macrophages may indicate a reduced susceptibility to the Fas/FasL-mediated apoptosis of lymphoid cells in *H pylori*-infection.

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Key words: Gastric ulcer; *H pylori*; Apoptosis; Fas receptor; Fas ligand; Perforin

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Abstract

AIM: To analyze the level of apoptosis in different mucosal compartments and the differential expression of Fas/Fas-ligand and perforin in *H pylori*-associated gastric ulcer.

METHODS: Antral specimens from patients with *H pylori*-related active gastric ulcer (GU), *H pylori*-related gastritis, and non-infected controls were analysed for densities and distribution of apoptotic cells determined by the TdT-mediated dUDP-biotin nick-end-labelling method. GU patients were submitted to eradication therapy with follow-up biopsy after 60 d. Fas, FasL, and perforin-expressing cells were assessed by immunoperoxidase, and with anti-CD3, anti-CD20 and anti-CD68 by double immunofluorescence and confocal microscopy. Quantitative analysis was performed using a computer-assisted image analyser.

RESULTS: *H pylori*-infected antrum showed greater surface epithelial apoptosis which decreased after eradication therapy. In the lamina propria, higher rates of mononuclear cell apoptosis were observed in *H pylori*-gastritis. Co-expression of Fas with T-cell and macrophage markers was reduced in GU. FasL- and perforin-

INTRODUCTION

Infection with *H pylori* induces chronic gastritis in virtually all infected hosts and it has been implicated as the major etiologic factor in peptic ulcer disease, chronic atrophic gastritis^[1,2], gastric carcinoma, and gastric lymphoma^[3,4]. An important feature of *H pylori* infection relies on the fact that the gastric mucosal immune response is not capable of eliminating the organism, and the infection persists indefinitely^[5]. The local inflammatory process is constituted by immunocompetent cells, including a large number of T cells in the lamina propria and an increased expression of T-helper type 1 (Th1) proinflammatory cytokines^[6,7].

H pylori has been reported to increase apoptosis of gastric epithelial cells which has been associated with gastric atrophy and also peptic ulcer^[8]. Although the mechanisms by which the infection with *H pylori* may

result in abnormal apoptosis are still unclear, there is some evidence indicating a major role for the host immune response. Results from an *in vitro* study demonstrated that interferon gamma (IFN-gamma) increases the attachment of the bacteria as well as the induction of apoptosis in gastric epithelial cell lines exposed to *H pylori*^[9]. In another *in vitro* study, proinflammatory stimuli such as tumor necrosis factor alpha, IFN-gamma, and a receptor-activating CD95/APO-1/Fas antibody, were shown to potentiate *H pylori*-induced apoptosis both directly and indirectly through sensitization of epithelial cells^[10].

Alterations in apoptosis have been associated with the pathogenesis of diseases such as cancer, viral infections, autoimmunity^[11], and inflammatory bowel disease^[12,13]. The increased apoptosis of epithelial cells triggered by *H pylori* has been related to the enhancement of the Fas/Fas ligand signaling pathway in the gastric mucosa^[14,15]. Results from *in vitro* studies have demonstrated a possible role for the Fas-mediated cytotoxicity of activated gastric T-cell lines in the apoptosis of gastric epithelial cells during the infection with *H pylori*^[16,17].

Apoptosis mediated by cytotoxic T lymphocytes (CTLs) is thought to constitute an essential mechanism of recognition of auto-reactive cells and of target-cell destruction. Although increased gastric epithelial apoptosis has been associated with the expansion of mucosal T-helper type 1 cells, the role of T-cell mediated cytotoxicity in that process is still unclear. Therefore, this study was designed to evaluate the rate of apoptosis in different mucosal compartments of *H pylori*-associated gastric ulcer disease. In addition, distinct pathways of cytotoxicity were assessed through the differential expression of Fas and Fas ligand, and of perforin in the gastric mucosa of patients in different clinical conditions including after eradication therapy.

MATERIALS AND METHODS

Patients

Fifteen consecutive patients with *H pylori*-positive active gastric peptic ulcers, and 14 *H pylori*-positive and 14 *H pylori*-negative patients both with endoscopically normal gastroduodenal mucosa were enrolled in this study. None of the patients studied had received any antibiotics, nonsteroidal anti-inflammatory drugs, bismuth compounds, H₂-receptor antagonists, proton pump inhibitors, anticancer drugs, or corticosteroids at least 6 mo before the beginning of the study. *H pylori* infection was confirmed by positive results in both rapid urease test and histological examination, while *H pylori*-negative patients were defined by the absence of bacteria in both tests.

Patients with gastric ulcers consisted of 7 women and 8 men, with a mean age of 44.4 years (range 18-71 years). All peptic ulcers were located in the antrum and lesions were at the Sakita A1-A2 stages. After biopsy specimens were obtained, all patients were submitted to eradication therapy consisting of a 2 wk course of lansoprazole 30 mg, amoxicillin 1 g, and clarithromycin 500 mg given twice daily. Control endoscopy with gastric biopsies was undertaken at least 60 d after completion of eradication therapy.

As controls, biopsy samples were obtained from

benign normal mucosa of consecutive patients undergoing diagnostic upper endoscopy for various clinical indications. Patients with *H pylori*-positive gastritis (without peptic ulcer) comprised 10 women and 4 men, with a mean age of 33.9 years (range: 20-58 years). The group of *H pylori*-negative patients consisted of 5 women and 9 men, with a mean age of 39.1 years (range: 20-63 years).

The study protocol was approved by the Ethical Committee of the University Hospital, Federal University of Rio de Janeiro, and informed consent was obtained from all patients.

Mucosal specimens

Gastric biopsy specimens were endoscopically obtained from the antrum (2 cm away from the pyloric ring) of all patients in the study. From patients with gastric ulcer biopsy samples were collected from the antrum both at the ulcer margins and at the opposite wall of the lesion.

Biopsy specimens were taken for urease test, histology, and immunohistochemical studies. One specimen was used for the urease test. Two specimens were fixed in 10% normal saline, embedded in paraffin, cut into 5 µm sections and stained with hematoxylin and eosin (HE) and with Giemsa for histopathological examination. Another two antral specimens were immediately covered with Tissue Tek O.C.T. compound (Miles Scientific Laboratories Ltd, Naperville, IL, USA) and snap frozen in isopentane in a liquid nitrogen bath. These specimens were subsequently stored at -80°C before processing, and cut into 5 µm sections in a cryostat maintained at -20°C. Tissue sections were mounted onto slides pre-treated with poly-L-lysine (Sigma Chemical Co., St Louis, MO, USA), air-dried and fixed for 10 min in acetone at room temperature.

Histologic analysis

Formalin-fixed gastric sections were stained with hematoxylin and eosin for the evaluation of gastritis and with Giemsa stain to detect *H pylori*. The histological assessment of inflammation was performed according to the updated Sidney system^[18]. Briefly, the parameters evaluated were inflammation (mononuclear leukocyte infiltration) and activity (polymorphonuclear neutrophil infiltration) of gastritis, density of *H pylori* colonization, atrophy, and intestinal metaplasia, which were graded as absent (0), mild (1), moderate (2), and severe (3), respectively.

Immunohistochemistry

For this set of experiments, frozen sections were used to characterize different cell subsets and for the study of apoptosis, which were performed by using the indirect immunoperoxidase technique.

Immunohistochemical staining was carried out using the following primary mouse monoclonal antibodies: anti-CD3 (1:50), anti-CD68 (1:50), anti-CD20 (1:50), anti-CD95/APO-1 (1:50) (Dako A/S, Glostrup, Denmark), anti-Fas ligand (1:1000), and anti-perforin (1:100) (Pharmin-gen, San Diego, CA, USA).

Briefly, frozen sections were immersed in 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. After being rinsed in phosphate buffered

saline (PBS) containing 0.5% Tween 20 for 10 min, tissue sections were incubated with non-immune horse serum for 30 min and, subsequently, with the respective monoclonal antibody in a humidified chamber overnight, at room temperature. Two sections from each sample were incubated with either PBS alone or mouse monoclonal IgG1 (concentration-matched) (Dako A/S, Glostrup, Denmark) and served as negative controls. After being rinsed in PBS for 10 min, all tissue sections were incubated for 30 min with a goat anti-mouse peroxidase conjugate (1:200) (Zymed Laboratories, Inc., San Francisco, CA, USA).

To determine apoptosis, fragmented DNA was stained by the terminal deoxynucleotidyltransferase (TdT)-mediated dUDP-biotin nick end labelling (TUNEL) assay, with the TACS™ TdT kit-*in situ* apoptosis detection kit (R&D Systems, Minneapolis, MN, USA). Frozen sections were first incubated with proteinase K solution for 15 min at room temperature, and then immersed in hydrogen peroxide to block endogenous peroxidase activity, as described above. After washing, slides were incubated with the TdT labeling buffer for 5 min. This step was followed by the incubation with the labeling reaction mix containing TdT enzyme for 1 h at 37°C. The biotinylated nucleotides incorporated into DNA fragments were detected using streptavidin horseradish peroxidase conjugate. A second section of each sample, incubated without TdT enzyme, constituted the negative controls. Positive controls were prepared by treating samples with TACS-nuclease.

After being rinsed in PBS, all sections were developed with a solution containing hydrogen peroxide and diaminobenzidine. Preparations were lightly counterstained in Harris's hematoxylin, dehydrated, and mounted in Permount (Fisher Scientific, Pittsburgh, PA, USA).

Morphologically preserved TUNEL-positive cells and apoptotic bodies were referred to as apoptotic cells and determined using predefined measurements in the computer-assisted image analyser in conjunction with careful evaluation of morphologic criteria.

Immunofluorescence and confocal microscopy

In a double direct or indirect immunofluorescence study, the frozen sections were incubated overnight at 4°C with 2.5% bovine serum albumin (BSA), 2.0% skimmed milk, 8.0% fetal calf serum (FCS) blocking buffer under shaking. The sections were rinsed once with PBS and 0.05% Tween 20 and then incubated with appropriately diluted primary antibodies in PBS and 1.0% FCS for 1 h in a humidified atmosphere at 37°C. The primary antibodies used were the anti-CD3 FITC (1:50), anti-CD68 FITC (1:50), anti-CD20 FITC (1:50), anti-CD95 (Fas/APO-1) R-PE (1:50) (Dako A/S, Glostrup, Denmark), anti-Fas ligand (1:1000), and the anti-perforin R-PE (1:100) (Pharmingen, San Diego, CA, USA). After rinsing three times in PBS containing 0.05% Tween 20 for 5 min each, tissue sections incubated with anti-Fas ligand were incubated for 1 h with TRITC-conjugated Fab fraction of goat anti-mouse IgG antibody (Dako A/S, Glostrup, Denmark), as appropriate for indirect immunolabelling. Slides were rinsed three times and mounted in an anti-fading medium containing buffered

glycerol and *p*-phenylenediamine (Sigma Chemical Co., St Louis MO, USA), and then observed with a Zeiss LSM 510 META confocal laser scanning microscope. At least four representative images from each slide were captured at distinct excitation wavelengths (488 nm for FITC conjugate, and 543 nm for TRITC or R-PE conjugates), creating excitation lambda stacks. Using the META detector, excitation lambda stacks were separated into individual images corresponding to the signal from distinct dyes, eliminating autofluorescence and crosstalk between fluorochromes. Two sections from each sample were incubated with either PBS alone or FITC- or TRITC-conjugated anti-mouse IgG antibody, and served as negative controls.

Quantitative assessment

Quantitative analysis of tissue sections (under light microscope at $\times 400$ magnification) and of captured images of immunofluorescence (under confocal laser scanning microscope) was carried out using a computer-assisted image analyser (Image-Pro Plus Version 4.1 for Windows, Media Cybernetics, LP, Silver Spring, MD, USA). Any epithelial and lamina propria cells exhibiting identifiable reactivity distinct from background were regarded as positive.

In the immunoperoxidase studies, percentages of the different cell subsets or apoptotic cells were defined by the number of immunoreactive cells in relation to total cells (immunoreactive and non-immunoreactive cells) in the lamina propria per millimetre squared (counted in at least 10 different areas), or in at least 500 epithelial cells in the crypts and in the surface epithelium of longitudinally sectioned gastric pits.

In the double immunofluorescence studies, the immunoreactive cells were counted in the lamina propria per millimetre squared. Co-localization was defined as the numbers of double-positive cells (yellowish colour) in relation to one of the single-positive cells (green colour), in the lamina propria per millimetre squared (counted in at least 4 different areas). Co-expression was further confirmed with confocal LSM co-localization tool (data not shown). Two independent observers who were unaware of the patients' data examined all tissue sections and captured images.

Statistical analysis

Statistical analysis was performed using the statistical software SPSS for Windows (Version 10.0.1, SPSS Inc., 1989-1999, USA). Statistical differences among the experimental groups were evaluated with the one-way ANOVA test in which pairwise multiple comparisons were carried out using the Dunnett's T3 test, and the Wilcoxon signed rank test for comparisons between patients with gastric ulcers (before and after treatment; gastric antrum and marginal zone of the ulcer). Correlations between the densities of positive cells measured by immunohistochemistry and the co-localization studies were assessed using the Spearman rank correlation coefficient. Values are expressed as medians (1st quartile, 3rd quartile). The level of significance was set at $P < 0.05$.

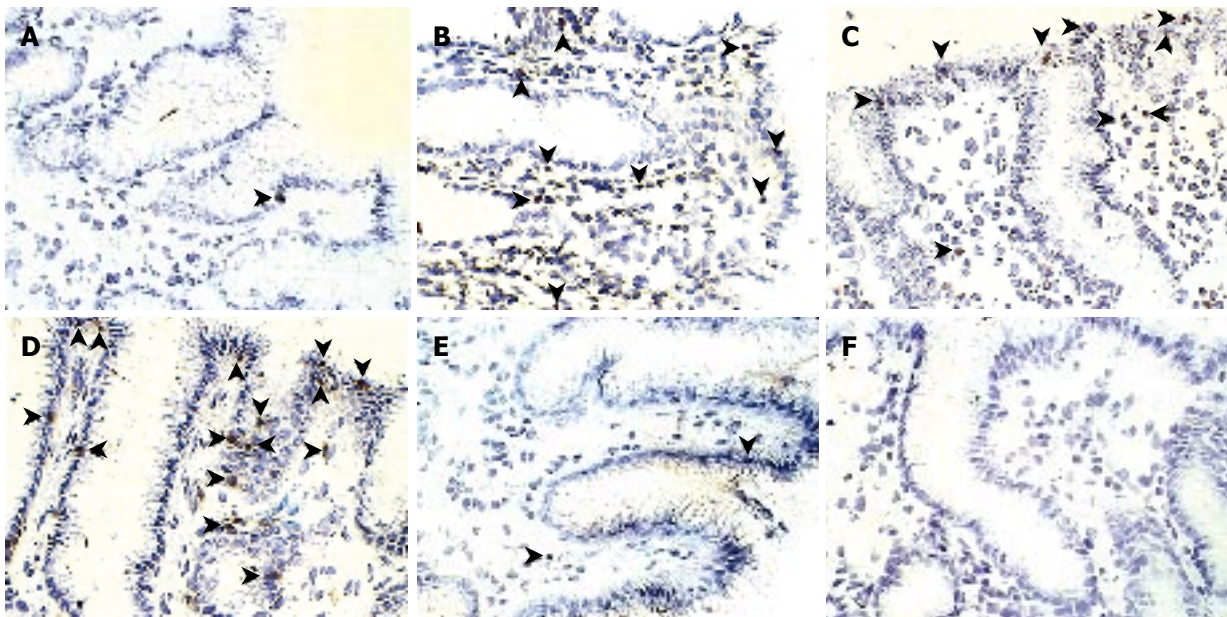


Figure 1 Terminal deoxynucleotidyltransferase (TdT)-mediated dUDP-biotin nick end labelling (TUNEL)-positive apoptotic cells. Photomicrographs show tissues obtained from the antral mucosa of: a non-infected control patient (A), a patient with *H. pylori*-associated gastritis (B), a patient with active gastric ulcer (C), at the ulcer margin of the same patient (D), and after eradication therapy (E), and a negative control, without TdT enzyme (F), respectively. Arrowheads show positive cells in the epithelium and in the lamina propria (Original magnification $\times 400$).

RESULTS

Inflammatory scores in gastric mucosa

Higher inflammatory scores were found in the mucosa of gastritis and GU patients infected with *H. pylori* compared to the normal mucosa of control patients free of infection and the mucosa of the same patients after eradication therapy. Scores in the GU patient group were 5.0 (3.3-5.0) at the ulcer margins, while the antrum scored 4.0 (3.0-5.0) before and 2.0 (1.0-2.0) after eradication therapy. In the antrum of untreated *H. pylori*-associated gastritis the score was 3.0 (3.0-3.0), and in the control group of normal non-infected mucosa the score was 0.5 (0.0-1.0). Atrophy and intestinal metaplasia were not detected in any specimens.

Proportions of T and B cells, and macrophages

A significantly higher number of T cells was found at both the ulcer margin and antrum of patients with GU (12.7% and 17.7%, respectively) compared with the normal non-infected control group (7.1%) ($P = 0.027$ and $P < 0.001$, respectively). After eradication therapy, rates of T cells in the antrum significantly decreased from 17.7% to 13.1% ($P = 0.046$). The number of macrophages was also significantly higher in the antrum of GU patients before treatment (12.0%) compared with that of the control group (6.9%) ($P = 0.029$). In addition, in the GU patient group, the number of macrophages in the ulcer margin and antrum (11.1% and 12.0%, respectively) decreased after eradication therapy (6.8%) ($P = 0.028$ and $P = 0.043$, respectively). In *H. pylori*-positive patients with gastritis, the number of T cells (13.9%) and macrophages (12.7%) was not significantly different from those in the GU and control group. The proportions of B cells were similar in all groups.

Proportions of apoptotic cells in the epithelium and lamina propria

In the GU group, the location of epithelial cell apoptosis was predominant in the superficial layer, both at the ulcer margin and the opposite wall of the antrum. In contrast, in the infected mucosa from patients with gastritis, apoptosis was more frequent in the crypts (Figure 1). When the proportion of apoptotic cells was assessed, patients who were *H. pylori*-positive contained a significantly ($P < 0.03$) greater number of apoptotic cells in the mucosa, regardless of the diagnosis, than in the same group after eradication and in non-infected controls (Figure 2). In the GU group, the number of apoptotic cells was significantly ($P < 0.003$) higher than in the gastritis group. The proportion of apoptotic cells in the crypts was similar in all groups.

When the lamina propria was assessed, apoptosis of inflammatory cells was seen more frequently ($P < 0.01$) in the mucosa of patients with gastritis compared to that of treated GU patients and non-infected controls (Figure 2). A significant correlation was found between the inflammatory scores and the number of apoptotic cells in the surface epithelium ($r = 0.71$, $P < 0.001$), but not the crypt epithelium or the lamina propria.

Expression of Fas and Fas L by gastric inflammatory cells

Immunohistochemistry revealed that Fas was constitutively expressed by all epithelial cells, with no differences detected among all groups. In the lamina propria, the percentage of Fas-positive cells was significantly increased in patients with GU, both at the ulcer margin and the antral mucosa, compared with the mucosa of the same patients after eradication therapy ($P < 0.01$) and those of non-infected controls ($P < 0.04$) (Figure 3). A significant correlation was

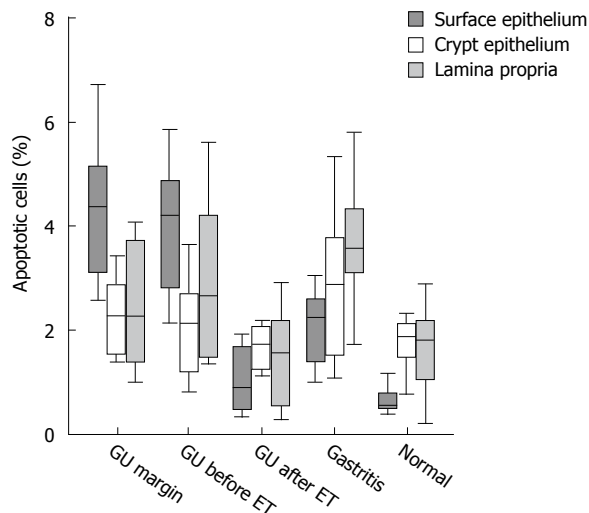


Figure 2 Percentages of apoptotic cells in the surface and crypt epithelium, and in the lamina propria of patients with gastric ulcer (GU) [at the ulcer margin, and the antrum before and after eradication therapy (ET)], *H pylori*-associated gastritis, and non-infected controls, respectively. Horizontal bars represent medians, boxes represent the 25th and 75th percentiles, and vertical bars represent ranges. In the surface epithelium values were significantly different compared to GU after ET ($P = 0.018$; $P = 0.003$; $P = 0.027$); gastritis ($P = 0.003$; $P = 0.002$), and the control group ($P < 0.001$; $P = 0.001$). In the lamina propria values were significantly different compared to GU after ET ($P = 0.001$), and the control group ($P = 0.001$). In the crypt epithelium values were not significantly different among groups (n : GU = 12, *H pylori*-associated gastritis = 11, control = 10). Differences were analysed using one-way ANOVA with the Dunnett's test for multiple comparisons. The Wilcoxon signed rank test was used for comparisons between GU patients.

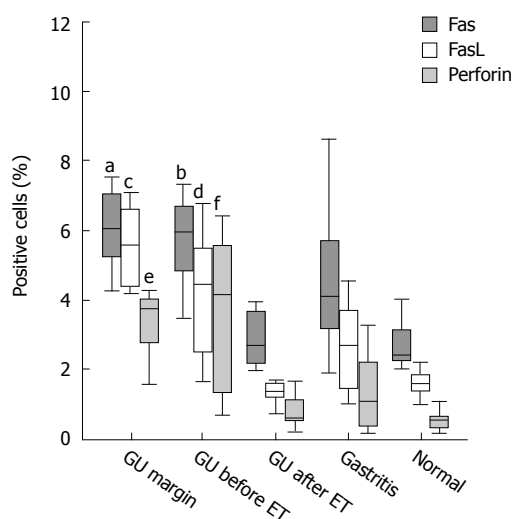


Figure 3 Quantitative analysis of apoptosis-related proteins in the gastric lamina propria of patients with gastric ulcer (GU) [at the ulcer margin, and the antrum before and after eradication therapy (ET)], *H pylori*-associated gastritis, and non-infected controls, respectively. Horizontal bars represent medians, boxes represent the 25th and 75th percentiles, and vertical bars represent ranges. Values of Fas were significantly different compared to the antral mucosa after ET ($^aP = 0.005$, $^bP = 0.008$), and the normal group ($^cP < 0.001$, $^dP = 0.031$). Values of FasL were significantly different compared to GU after ET ($^eP = 0.003$, $^fP = 0.008$), gastritis ($^gP < 0.001$), and the control group ($^hP < 0.001$, $^iP = 0.018$). Values of perforin were significantly different compared to GU after ET ($^jP = 0.005$, $^kP = 0.008$), gastritis ($^lP = 0.004$, $^mP = 0.033$), and the control group ($^nP = 0.001$, $^oP = 0.006$) (n : GU = 12, *H pylori*-associated gastritis = 11, control = 10). Differences were analysed using one-way ANOVA with the Dunnett's test for multiple comparisons. The Wilcoxon signed rank test was used for comparisons between GU patients.

found between the number of Fas-positive cells within the lamina propria and inflammatory scores ($r = 0.62$, $P < 0.001$).

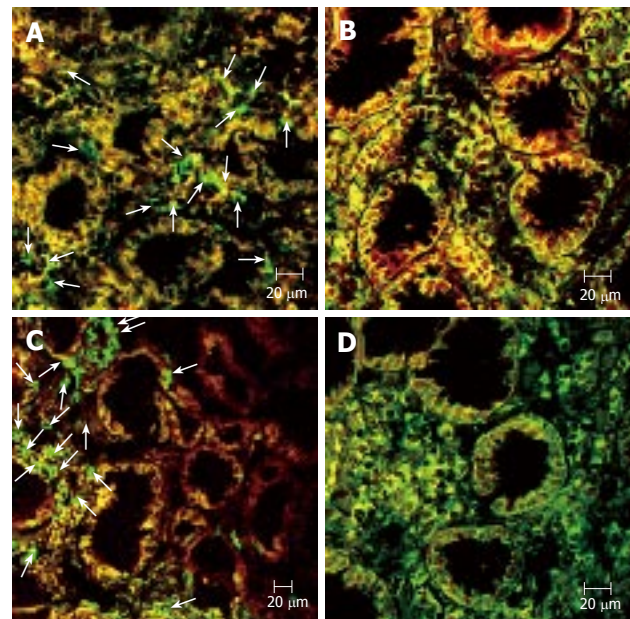


Figure 4 Double immunofluorescence analysis. Expression of CD3 (FITC) and Fas (R-PE) in the ulcer margin of a patient with gastric ulcer (A) and a non-infected control (B). Expression of CD68 (FITC) and Fas (R-PE) in the antrum of a patient with gastric ulcer (C) and a patient with *H pylori*-related gastritis (D). Images obtained by confocal microscopy. Arrows show CD3 or CD68 (FITC)-single-positive cells (green) without correspondent Fas (R-PE)-positive cells in the lamina propria of the same tissue section.

Next, we tried to identify the type of inflammatory cells co-expressing Fas by confocal double immunofluorescence. The expression of Fas by lamina propria T cells from the ulcer margin and the untreated antral mucosa was significantly reduced compared with both cells in the antrum after treatment ($P < 0.05$) and in non-infected controls ($P < 0.02$) (Figures 4 and 5). Essentially the same results were obtained when co-expression of Fas by macrophages was evaluated (Figures 4 and 5). In regard to B cells, no significant differences were found in the gastric mucosa among all groups.

Immunostaining for FasL was predominantly located in the lamina propria, and rarely seen in intraepithelial lymphocytes. The percentage of FasL-positive cells was increased in the mucosa of patients with GU, both at the ulcer margin and the antrum before treatment, compared with that observed after treatment and in the non-infected control group (Figures 3 and 6). We further investigated the co-localization of FasL in the gastric lamina propria and, as expected, essentially all FasL-positive cells co-expressed CD3 (data not shown). Of note, a significant correlation existed between the expression of FasL by gastric mucosa T cells and the percentages of apoptotic cells in the surface epithelium ($r = 0.71$; $P < 0.001$) and the histological inflammatory scores ($r = 0.63$, $P < 0.001$).

Expression of perforin by gastric inflammatory cells

Immunostaining for perforin was essentially restricted to the lamina propria, with occasional staining in intraepithelial lymphocytes. Perforin-positive cells were significantly more frequent in the lamina propria of patients with GU, both at the ulcer margin and the untreated antrum, compared to the antrum of gastritis patients ($P < 0.04$),

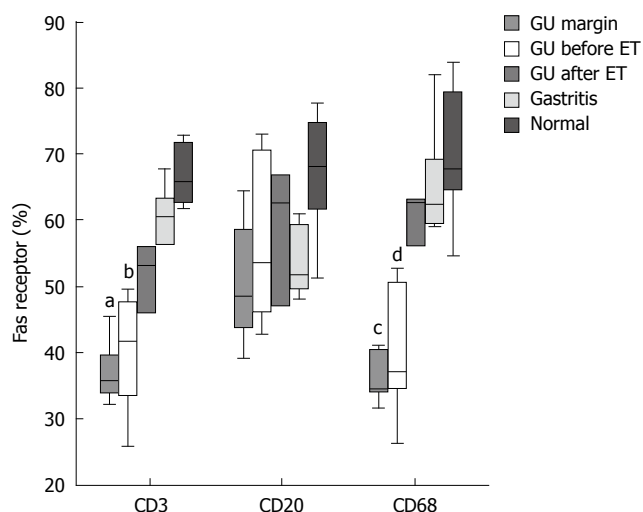


Figure 5 Percentages of the expression of Fas by CD3, CD20, and CD68 positive cells in the antral lamina propria of patients with gastric ulcer (GU) [at the ulcer margin, and at the antrum before and after eradication therapy (ET)], patients with *H pylori*-associated gastritis, and from non-infected controls, respectively. Horizontal bars represent medians, boxes represent the 25th and 75th percentiles, vertical bars represent ranges. In regard of the expression of Fas with CD3 values were significantly different compared to GU after ET (^a $P = 0.043$), gastritis (^b $P = 0.021$); and the control group (^c $P < 0.001$; ^d $P = 0.012$). For Fas expression with CD20 values were not significantly different among groups. For Fas expression with CD68 values were significantly different compared to GU after ET (^e $P = 0.043$), gastritis (^f $P = 0.007$; ^g $P = 0.034$); and the control group (^h $P = 0.012$; ⁱ $P = 0.027$) ($n = 6$, in each group). Differences were analysed using one-way ANOVA with the Dunnett's test for multiple comparisons. The Wilcoxon signed rank test was used for comparisons between GU patients.

treated GU patients ($P < 0.01$), and normal non-infected controls ($P < 0.01$) (Figures 3 and 6). Confocal microscopy confirmed that all perforin-positive cells were also CD3-positive (data not shown). A significant correlation was detected between the number of perforin-expressing cells in the gastric mucosa and the percentage of apoptotic cells in the surface epithelium ($r = 0.61$; $P < 0.001$) as well as the inflammatory scores ($r = 0.59$, $P < 0.001$).

DISCUSSION

In the current study, we detected increased rates of apoptosis in the surface epithelium of the gastric mucosa infected with *H pylori*, predominantly of GU patients. Increased rates of apoptosis were also observed among lamina propria mononuclear cells of patients with *H pylori*-associated gastritis. The expression of Fas, FasL, and perforin in the *H pylori*-infected gastric mucosa was increased compared with normal non-infected controls and with patients after eradication therapy. The higher expression of apoptosis-related molecules was found in the antral mucosa of patients with gastric ulcer, and this result was significantly correlated with the levels of apoptosis at the surface epithelium. In addition, we showed for the first time an *in situ* analysis of the densities and co-localization of apoptosis-related molecules in the antral mucosa from patients with gastric ulcer, before and after eradication therapy, and both at the ulcer margin and at the opposite antral wall. The results demonstrated that there was a reduction in the expression of Fas by both T cells and macrophages in the antral lamina propria of GU

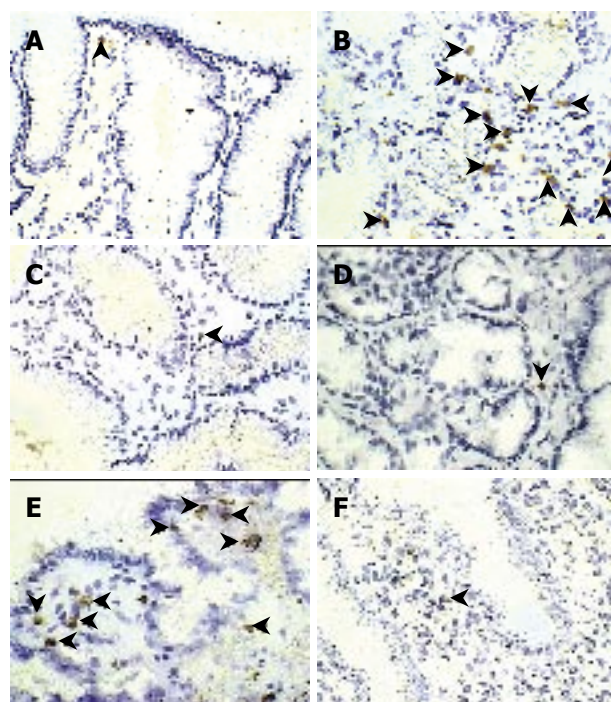


Figure 6 Apoptosis-related proteins in the gastric mucosa stained with immunoperoxidase. Photomicrographs show the antral mucosa stained for FasL in a non-infected control patient (A), at the ulcer margin (B), and after eradication therapy of the same patient (C). Immunostaining for perforin is shown in a patient with *H pylori*-associated gastritis (D), and in a patient with gastric ulcer before (E), and after eradication therapy (F), respectively. Arrowheads show immunoreactive cells (Original magnification $\times 400$).

patients.

Although *H pylori*-related gastritis continues to constitute one of the most common human infections, only a subset of patients will develop severe complications of the infection such as peptic ulcer disease. Histological findings of the chronically infected stomach reveal a dense cellular infiltration throughout the mucosa, whereas *H pylori* locates predominantly in the overlying mucous layer. Several studies have provided evidence that activated T-helper type 1 cells are increased in *H pylori*-associated gastritis^[6,7], and the consequent pro-inflammatory stimuli have been associated with the sensitization and induction of epithelial apoptosis^[9,10]. However, in normal conditions epithelial apoptosis appears to be an infrequent event in the gastric mucosa. In the present work, the results of *in situ* analysis suggest that increased rates of epithelial apoptosis are related to *H pylori* infection, in agreement with previous *in vivo* and *in vitro* studies^[8,19,20]. Significantly higher rates were found in patients with peptic ulcer and decreased significantly after eradication therapy reaching normal levels. In addition, the localization of epithelial apoptosis differed among groups. In the peptic ulcer group apoptotic cells were found predominantly in the superficial epithelium, whereas in the gastritis group the phenomenon was mostly located at the crypts. Distinct localization patterns of epithelial apoptosis seem to be in accordance with either the most likely outcome of peptic ulcer whenever surface epithelial damage prevails or with atrophy in chronic *H pylori*-associated gastritis.

The induction of macrophage apoptosis has been

considered as a possible method employed by multiple pathogens to escape the host immune response^[21]. In fact, apoptosis of immunocompetent cells such as macrophages^[22], and B cells^[23] has been reported in association with *H pylori* infection. Furthermore, *H pylori* may also evade the host immune response through the impairment of antigen-specific T cell proliferation^[24], or inducing T cell apoptosis^[25]. Therefore, at least in theory, apoptosis of immunocompetent cells is likely to decrease the local inflammatory process, but on the other hand it may reduce the effective immune response against *H pylori* as well. Apoptosis of mononuclear leukocytes in the gastric mucosa infected by *H pylori* was also reported after eradication therapy^[26]. Although in the present study the rates of apoptosis in the lamina propria after eradication therapy were comparable to normal controls, the mucosa showed a concomitant reduction in the number of inflammatory cells. Thus, it was possible that the number of those cells could have been reduced by induction of apoptosis after therapy. However, if this was the case in our study, the effect would be likely to have decreased to the normal levels by the time of the follow-up biopsy. On the other hand, a relative resistance to *H pylori*-induced apoptosis was detected in neutrophils^[27], and dendritic cells^[28] *in vitro*. In this study, we found significantly increased rates of apoptosis in the lamina propria mononuclear cells of patients with *H pylori*-related gastritis compared to the non-infected mucosa. The fact that this phenomenon has not been observed in the antral mucosa of GU patients may reflect the existence of a defect in the control of local host responses. It is possible that apoptosis of lamina propria mononuclear cells might constitute a mechanism by which the host may down-regulate the inflammatory response associated with chronic *H pylori* infection.

Apoptosis constitutes the primary mechanism of target-cell destruction mediated by cytotoxic T lymphocytes, and one of the pathways involved in this process is the Fas/FasL. Increased rates of apoptosis reported in *H pylori*-infected gastric mucosa has been associated with the Fas/FasL pathway^[15,17,29]. *In vitro* studies have demonstrated that the sensitivity of gastric epithelial cells to Fas-mediated apoptosis could be increased by the up-regulation of the Fas-receptor expression consequent to *H pylori* infection^[14] or triggered by inflammatory cytokines^[30]. In addition, it was demonstrated that increased expression of Fas by gastric epithelium renders cells sensitive to apoptosis by adjacent FasL-expressing T cells^[16].

In contrast to T cell responses, the epithelial expression of FasL is low or undetectable^[31], and blocking the Fas receptor cannot protect those cells from apoptosis^[29]. Therefore, it seems that *H pylori* may be capable of inducing apoptosis in different cell lineages using diverse but possibly overlapping mechanisms. Nevertheless, in T cells the response appears to be mostly dependent on Fas/FasL interactions^[25]. In the present study, we found that the levels of FasL-positive cells in the lamina propria were positively correlated with surface epithelial apoptosis. However, increased numbers of FasL-positive cells were present in all patients infected with *H pylori*, without any significant difference between the results of gastritis and

GU patients. These findings suggest that FasL might participate in the inflammatory process associated with *H pylori* infection, but may not be directly and specifically related to ulcerogenesis.

Fas/FasL-mediated apoptosis is regulated mostly by the cellular expression of both molecules, since Fas is activated almost exclusively by the membrane-bound form of its natural ligand, FasL^[32]. However, cellular mechanisms that control the expression of Fas and FasL appear to be independent. Fas is more universally distributed and is thought to be expressed later in the course of cellular activation^[33], while the up-regulation of FasL is regarded as an early event of T-cell activation^[34]. Hence, the expression of Fas on T-cells and other lamina propria mononuclear cells could have an effect of down-regulation of gastrointestinal immune responses through apoptosis, upon binding with FasL. In accordance with the findings of the present study, Fas is expressed constitutively in the epithelial cells of the human stomach^[30], where it has been considered as a key molecule in the control of apoptosis. In the lamina propria infected with *H pylori* we observed increased numbers of Fas-expressing cells. However, since the numbers of inflammatory cells were also significantly increased in the infected mucosa, the expression of Fas could actually be proportionally altered. In fact, the expression of Fas was shown to be reduced by both T cells and macrophages in the lamina propria of GU patients. Taken together, these results suggest that the increased numbers of FasL-expressing cells might result in increased epithelial cytotoxicity not paralleled by lamina propria mononuclear cell apoptosis mediated by the Fas/FasL system in GU patients. It is hypothesized that an uncoordinated regulation of Fas and FasL could possibly result in decreased apoptosis of inflammatory cells, including auto-reactive cells in the lamina propria of GU patients.

In conjunction with T cells, macrophages are recognized as fundamental elements of the chronic inflammatory process of GU. Therefore, it is reasonable to suppose that mechanisms to eliminate activated macrophages should also be required for the maintenance of peripheral tolerance. Results from an *in vitro* study demonstrated that in addition to T-T and T-B lymphocyte cytotoxicity, the Fas/FasL system is also capable of mediating T-macrophage cytotoxicity^[35]. Hence, in the current study the reduced expression of Fas on macrophages shown in the gastric mucosa from patients with gastric ulcer could possibly explain the accumulation and persistence of those cells in sites of gastric inflammation.

Increased levels of granzyme B mRNA, introduced through the action of perforin, have been found in cells adjacent to apoptotic epithelial cells of specimens obtained from the marginal zone of active gastric and duodenal ulcers. But interestingly, in that study the findings were shown also in patients with peptic ulcer in the absence of *H pylori* infection^[26]. In the current study, perforin was detected at increased rates in GU patients, both at margins and tissues away from the lesion, compared to both non-infected controls and *H pylori*-related gastritis. Moreover, the levels of perforin were significantly correlated with the

rates of surface epithelial apoptosis. This fact may support the contribution of distinct pathways to the cell-mediated cytotoxicity against innocent bystander targets such as the gastric epithelium, but possibly with a greater role for perforin in the development of peptic ulcer disease. On the other hand, the positive correlation between the rates of perforin-expressing cells and apoptotic cells in the lamina propria of patients with *H pylori*-associated gastritis may indicate the enhancement of an alternative pathway as a mechanism to circumvent the resistance to apoptosis and chronic mucosal inflammation.

In conclusion, the results indicate that increased apoptosis in the surface epithelium may contribute to the pathologic process of gastric ulcer. The lamina propria Fas/FasL expression may reflect the state of inflammation secondary to *H pylori* infection. However, the consistently increased percentage of perforin-expressing cells in the antrum of GU patients suggests a distinct role of perforin in ulcerogenesis.

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BASIC RESEARCH

An herbal formula, CGX, exerts hepatotherapeutic effects on dimethylnitrosamine-induced chronic liver injury model in rats

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regulated expression of TNF- α , TGF- β , TIMP-1, TIMP-2, PDGF- β , and MMP-2. Of these, the gene expression encoding PDGF- β and MMP-2 was still further enhanced 2 wk after secession of the 4-wk DMN treatment, and was remarkably ameliorated by CGX administration.

CONCLUSION: CGX exhibits hepatotherapeutic properties against chronic hepatocellular destruction and consequential liver fibrosis.

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Key words: Dimethylnitrosamine; Hepatotherapeutic; Herbal medicine; PDGF- β ; Chunggan extract

Shin JW, Son JY, Oh SM, Han SH, Wang JH, Cho JH, Cho CK, Yoo HS, Lee YW, Lee MM, Hu XP, Son CG. An herbal formula, CGX, exerts hepatotherapeutic effects on dimethylnitrosamine-induced chronic liver injury model in rats. *World J Gastroenterol* 2006; 12(38): 6142-6148

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Abstract

AIM: To evaluate the therapeutic effect of Chunggan extract (CGX), a modified traditional Chinese hepatotherapeutic herbal, on the dimethylnitrosamine (DMN)-induced chronic liver injury model in rats.

METHODS: Liver injuries were induced in Wistar rats by injection of DMN (ip, 10 mg/mL per kg) for 3 consecutive days per week for 4 wk. The rats were administered with CGX (po, 100 or 200 mg/kg per day) or distilled water as a control daily for 4 wk starting from the 15th d of the DMN treatment. Biochemical parameters (serum albumin, bilirubin, ALP, AST and ALT), lipid peroxides, hydroxyproline, as well as histological changes in liver tissues were analyzed. In addition, gene expression of TNF- α , TGF- β , TIMP-1, TIMP-2, PDGF- β , and MMP-2, all of which are known to be associated with liver fibrosis, were analyzed using real-time PCR.

RESULTS: CGX administration restored the spleen weight to normal after having been increased by DMN treatment. Biochemical analysis of the serum demonstrated that CGX significantly decreased the serum level of ALP ($P < 0.05$), ALT ($P < 0.01$), and AST ($P < 0.01$) that had been elevated by DMN treatment. CGX administration moderately lowered lipid peroxide production and markedly lowered hydroxyproline generation caused by DMN treatment in accordance with histopathological examination. DMN treatment induced a highly up-

INTRODUCTION

Several pathologic conditions, including chronic alcohol consumption, viral B or C hepatitis, and constant cholestasis, induce chronic injury to liver tissue. Moreover, these chronic liver disorders lead progressively to fibrosis or liver cirrhosis, which is an extreme form of chronic liver disease that reduces the quality of life and life span of the patients^[1]. Therefore, a crucial step in treating chronic liver disease involves preventing the development of cirrhosis. One of the best therapeutic strategies involves removing the underlying causes of liver damage^[2], and major findings on the epidemiological and pathogenic features that involve various cytokines or the transformation of stellate cells in the progression of chronic liver disease have recently been reported^[3-5]. Currently, however, no universally effective therapeutics exist, and chronic liver disease remains a major concern for global public health.

Medical research on natural products for treating chronic liver disease has been increasing in recent years^[6-8]. Chunggan extract (CGX, also called *Qinggan* extract, which means "cleaning liver") is a modified herbal drug based on a traditional Chinese hepatotherapeutic formula. It has therapeutic properties particularly for hyperlipidemia,

alcohol-induced liver damages in animal models, and other chronic liver diseases, including chronic viral B type hepatitis in clinical tests^[9-11]. Nevertheless, further studies are needed to evaluate the pharmaceutical efficacy based on scientifically controlled experiments before an appropriate clinical application can be implemented for treating various liver diseases.

We herein investigated the therapeutic effect of this drug on a chronic liver injury model in rats caused by dimethylnitrosamine (DMN).

MATERIALS AND METHODS

Components of CGX and fingerprinting analysis

The ingredients of CGX include 5 g each of *Artemisia capillaris* Herba, *Trionycis* Carapax, *Raphani* Semen; 3 g each of *Atractylodis Macrocephalae* Rhizoma, *Poria*, *Alismatis* Rhizoma, *Atractylodis* Rhizoma, *Salviae Miltiorrhizae* Radix; 2 g each of *Polyporus*, *Amomi* Fructus, *Aurantii* Fructus, and 1 g of *Glycyrrhizae* Radix or *Helenii* Radix. The 10.71% (w/w) lyophilized water-extract was obtained from the initial dried mixture according to the Korean standard over-the-counter (OTC) monographs, and its high-performance thin layer chromatography (HPTLC)-based fingerprint was produced by the CAMAG application system (Muttenez, Switzerland) (Figure 1A and 1B). For the HPTLC analysis, water extracts of CGX, *Artemisia capillaris* Herba, *Aurantii* Fructus, and their standard components, 6, 7-dimethoxycoumarin (Sigma Chemical Co., St. Louis, MO, USA) and Poncirin (Fluka, St. Louis, MO, USA), were dissolved in HPLC-grade methanol and applied to pre-washed silica gel 60 F254 HPTLC plates (size 10 cm × 10 cm, thickness of the silica gel 0.2 mm; Merck, Darmstadt, Germany) with an automated applicator (Linomat IV; CAMAG). The samples were then separated (migration distance 75 mm) using HPLC-grade ethyl acetate/formic acid/acetic acid/water (15:1:1:2). The migrated components were visualized under UV radiation at 366 nm using Reprostar 3 with a digital camera (CAMAG).

Animals and reagents

Specific pathogen-free 4-wk-old male Wistar rats were purchased from a commercial animal breeder (Samtako, Osan, Korea). Forty rats were acclimated for 3 wk and housed in an environmentally controlled room at 22 ± 2°C, 55% ± 10% relative humidity, and 12-h light/dark cycles, and fed commercial pellets (Samtako) and tap water *ad libitum*. Animal experiments were conducted in accordance with the Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Dimethylnitrosamine (DMN, *N*-Nitrosodimethylamine) and other reagents, including hydroxyproline, *p*-dimethylaminobenzaldehyde, and chloramine-T were purchased from Sigma, and perchloric acid was obtained from GFS Chemical Co. (Columbus, OH, USA).

Induction of chronic liver damage and administration of CGX

Forty Wistar rats with average body weight 210 ± 15 g were randomly divided into 5 groups (8 rats per group) as follows: naive (no treatment); DMN 4 wk (4-wk DMN treatment and sacrificed following the treatment

period); control (4-wk DMN treatment and 4-wk water administration from the third week of DMN treatment); and CGX 100 or CGX 200 (4-wk DMN treatment and 4-wk CGX administration from the third week of DMN treatment). DMN diluted in saline was intraperitoneally injected at 10 mg/mL per kg for 3 consecutive days during each week for the DMN 4 wk, control, CGX 100, and CGX 200 groups. The DMN 4 wk group was sacrificed at the end of the 4-wk DMN treatment. The CGX 100, CGX 200, and control groups were administered CGX with (100 or 200 mg/kg per day) or distilled water as a control daily for 4 wk starting from the third week of DMN-treatment. This experimental model is presented in Figure 1C.

Measurement of the relative organ weights, and histomorphologic examination of the liver

The changes in body weight for all animals were recorded weekly throughout the experimental period. The animals were sacrificed on the last day of experimentation or following the 4-wk induction for the DMN-4 wk group; the liver and spleen were removed and weighed to determine changes in the organ weights by treatment. The liver was removed and separately stored for histomorphologic examination, RNA expression analysis, as well as hydroxyproline and lipid peroxide determination.

For the histomorphological evaluation, a portion of liver tissue was fixed in Bouin's solution. The paraffin-embedded liver was sectioned (4-μm thickness), and Hematoxylin & eosin staining and Masson's trichome staining were performed. The representative histopathological features, such as necrosis or inflammatory cell infiltration and fibrosis, were examined under microscopy.

Complete blood count and serum biochemical analysis

The rats were bled via abdominal aorta after ether anesthesia on the final day of the 4-wk induction for the DMN 4 wk group or on the final day of full experimentation for others. Complete blood counts were performed by blood cell counter (HEMAVET; CDC Technologies Inc., Oxford, CT, USA) using small amounts of blood mixed in EDTA.

Once sera were prepared from the remaining blood, the levels of total protein, albumin, total bilirubin, alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase (ALT) were determined in the serum using an Auto Chemistry Analyzer (Chiron Ltd., Emeryville, CA, USA).

Lipid peroxidation measurement by malondialdehyde determination in liver tissues

Lipid peroxidation in liver tissues was examined using the method of thiobarbitric acid reactive substances (TBARS) as previously described^[12]. The concentration of TBARS was expressed as n moles of malondialdehyde (MDA) per milligram of tissue using 1.1.3.3-tetraethoxypropane (TEP) as a standard. Briefly, 0.2 g of liver tissue was homogenized in 2 mL of ice-cold 11.5 g/L KCl; then 0.13 mL of homogenate was mixed with 0.08 mL of 10 g/L phosphoric acid and 0.26 mL of 0.67% thiobarbituric acid. After heating the mixture for 45 min at 100°C, 1.03 mL of *n*-butanol was added into the mixture, followed

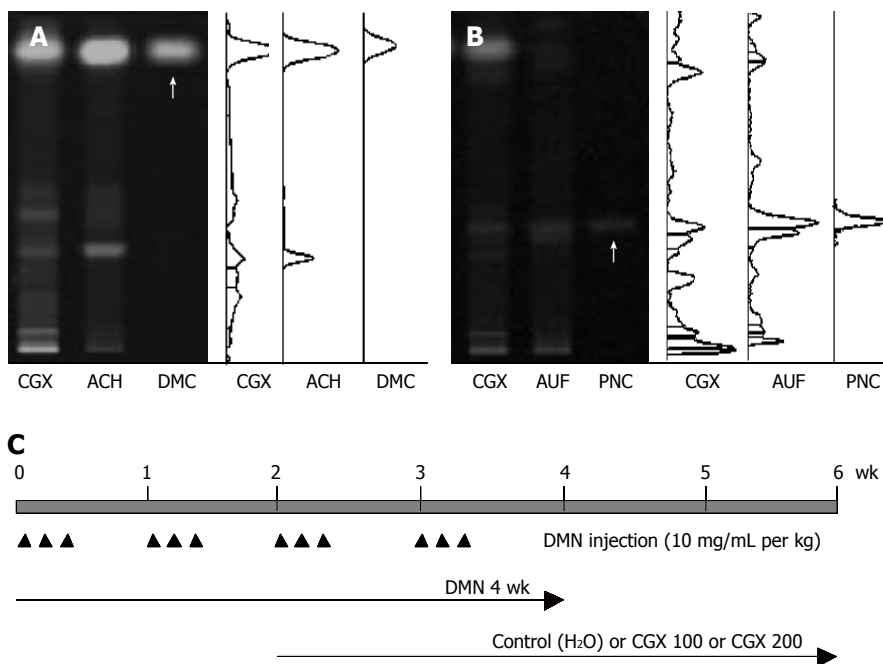


Figure 1 HPTLC-based fingerprint for CGX and scheme for the experiment. HPTLC analysis was performed to characterize CGX and its two major components (extracts from *Artemisia capillaris* Herba and *Aurantii Fructus*) with reference components, 6,7-dimethoxycoumarin and Poncirin. **A:** 2 µL of CGX (200 mg/mL) and 1 µL of the extract from *Artemisia capillaris* Herba (50 mg/mL) were subjected to HPTLC with 1 µL of 6, 7-dimethoxycoumarin (0.1 mg/mL); **B:** 2 µL of CGX (200 mg/mL) and 1 µL of the extract from *Aurantii Fructus* (50 mg/mL) were subjected to HPTLC with 1 µL of Poncirin (5 mg/mL). Arrows indicate 6,7-dimethoxycoumarin and Poncirin, respectively; **C:** Simplified scheme summarizing the experimental design: DMN treatment (ip, 3 consecutive days per week, 10 mg/mL per kg for 4 wk) and CGX administration (po, 100 or 200 mg/mL per kg, for 4 wk from 2 wk after DMN treatment). ACH: *Artemisia capillaris* Herba; AUF: *Aurantii Fructus*; DMC: 6,7-dimethoxycoumarin; PNC: Poncirin.

by vigorous mixing and centrifugation at 3000 r/min for 15 min. The absorbance of the upper organic layer was measured at 535 and 520 nm with a spectrophotometer, and compared to the value from freshly prepared TEP as a standard.

Collagen measurement by hydroxyproline determination in liver tissues

Hydroxyproline assays were performed as previously described with a slight modification^[13]. Briefly, liver tissues (0.2 g) stored at -70°C were homogenized in 2 mL of 6 mol/L HCl and incubated overnight at 110°C. After filtering the acid hydrolysates using filtering paper (Toyo Roshi Kaisha, Tokyo, Japan), 50 µL of samples and standard hydroxyproline in 6 mol/L HCl were incubated to dry out the HCl; 50 µL of methanol, 1.2 mL of 500 mL/L isopropanol, and 200 µL of chloramine T solution were sequentially added to the samples, followed by incubation at room temperature for 10 min. Next, 1.3 mL of Ehrlich's solution was added to the mixtures and incubated further at 50°C for 90 min. At the end of incubation, absorbance of the reaction mixtures was read at 558 nm. A standard curve was constructed using 0-1.0 mg/50 µL of hydroxyproline solutions.

Gene expression analysis using real-time PCR

Total RNA was extracted from liver tissue samples with Trizol (Invitrogen, Carlsbad, CA, USA), RNeasy (Ambion, Inc., Austin, TX, USA), and RNeasy column (Qiagen, Inc., Valencia, CA, USA). Complementary DNA (cDNA) was synthesized using 10 pmol of oligo dT and 10 pmol/L of random hexamer (Bioneer, Daejeon, Korea). After cDNA synthesis, quantitative real-time PCR was performed using SYBR green supermix reagent (Bio-Rad, Hercules, CA, USA) with the primers (forward and reverse, respectively) for β -actin: 5'-GTGGGGCGCCCCAGGCACCA-3' and 5'-CTCCTTAATGTCACGCACGATTTC-3'; tumor

necrosis factor- α (TNF- α): 5'-CTCCCAGGTTCTC TTCAAGG-3' and 5'-TGGAAGACTCCTCCAGGTA; transforming growth factor- β (TGF- β): 5'-TGAGTG GCTGTCTTTTGACG-3' and 5'-TTCTCTGTGGAGCTGAAGCA; platelet-derived growth factor beta (PDGF- β): 5'-GAGTCGAGTCGG AAAGCTCA-3' and 5'-CTGCTGCAGCCAGAGACC; matrix metalloproteinase-2 (MMP-2): 5'-GCGCTTT TCTCGAATCCAT-3' and 5'-GGGTATCCATCTCC ATGCTC-3'; tissue inhibitor of metalloproteinase-1 (TIMP-1): 5'-CGGACCTGGTTATAAGGGCT-3' and 5'-ACTCTCCAGTTTGCAAGGGA; and tissue inhibitor of metalloproteinase-2 (TIMP-2): 5'-GCATCACCAG AAGAAGAGC-3' and 5'-GTTTCCAGGAAGGGATG TCA-3'. Reactions were performed with 12.5 µL of iQ SYBR green supermix, 1 µL of 10 pmol/L primer pairs, 10.5 µL of distilled water, and 1 µL of cDNA. Each PCR run was performed under the following conditions: initial denaturation at 95°C for 5 min, 40 amplification cycles including denaturation at 95°C for 1 min, annealing at 58°C for 40 s, and elongation at 72°C for 40 s, followed by a single fluorescence measurement.

Statistical analysis

The results were expressed as the mean \pm SD. Statistical analysis of the data was carried out using Student's *t* test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Relative liver and spleen weights

As shown in Figure 2, 4-wk DMN treatment decreased relative liver weights compared to the control group, while it increased relative spleen weight. A 2-wk cessation of DMN following the 4-wk DMN treatment slightly restored these changes as seen in the control groups. These restorations were augmented by CGX administration (200 mg/kg), especially the spleen weights, compared to the

Table 1 Hematological analysis

Groups	Naive	DMN 4wk	Control	CGX 100	CGX 200
Erythrocyte (10 ⁶ /μL)	8.0 ± 0.3	7.1 ± 0.9	7.5 ± 1.5	8.4 ± 0.4	8.2 ± 0.5
Hemoglobin (g/dL)	15.4 ± 0.8	13.5 ± 1.3	14.0 ± 2.3	14.9 ± 0.6	14.8 ± 0.4
Hematocrit (%)	41.9 ± 1.4	35.2 ± 2.9	40.0 ± 6.1	40.9 ± 1.8	41.2 ± 2.6
Platelets (10 ³ /μL)	596 ± 459	449 ± 170	848 ± 265	795 ± 357	502 ± 305
Leukocytes (10 ³ /μL)	4.8 ± 1.2	4.9 ± 2.0	4.2 ± 1.8	2.6 ± 1.0	3.3 ± 1.2
Neutrophil (%)	17.1 ± 3.7	50.5 ± 7.1	39.7 ± 18.5 ^b	32.2 ± 9.1	34.7 ± 20.2
Lymphocyte (%)	79.2 ± 3.7	44.2 ± 6.0	53.3 ± 18.4 ^b	63.9 ± 10.1	61.1 ± 19.2
Monocytes (%)	3.6 ± 0.8	4.7 ± 1.7	6.6 ± 1.5 ^c	3.6 ± 2.0 ^a	3.8 ± 1.3 ^a
Eosinophil (%)	0.1 ± 0.1	0.4 ± 0.3	0.4 ± 0.4	0.3 ± 0.2	0.3 ± 0.3
Basophil (%)	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.2	0.1 ± 0.0	0.1 ± 0.2

After just 4-wk DMN treatment (DMN 4 wk), and after 6-wk full experiment for or control group including naïve group, complete blood count analysis was performed using EDTA-mixed blood. ^a*P* < 0.05 vs control group; ^b*P* < 0.05, ^c*P* < 0.01 vs naïve group.

control group (*P* < 0.05; Figure 2).

Hematological analysis

DMN treatment for 4 wk decreased the level of erythrocytes, hemoglobin, hematocrit and platelets, and decreased the lymphocyte population in peripheral blood compared to the naïve group (Table 1). Most of these hematological changes, however, had almost completely recovered to normal levels by cessation of the treatment for 2 wk (control group). These hematological improvements may, therefore, not have been due to the effect of CGX, with the exception of the decreased monocyte population of leukocytes in CGX 100 and CGX 200 groups (*P* < 0.05).

Serum biochemical analysis

Four-week treatment with DMN induced liver dysfunction, including lowered levels of total protein and albumin along with elevated level of total bilirubin, ALP, AST, and ALT (Figure 3A-3C). Although these biochemical parameters were partially restored after the 2-wk cessation period in the control group, AST and ALT levels still differed significantly from their normal values.

CGX treatment (100 mg/kg) showed significant restoring effects on serum level of total proteins and albumin (*P* < 0.01 and *P* < 0.05, respectively; Figure 3A). However, bilirubin, which was rapidly reduced by cessation of DMN treatment for 2 wk, was not affected by CGX administration. The elevated ALP level significantly decreased with treatment by both concentrations of CGX (Figure 3B), and the CGX treatment significantly lowered the of AST back to nearly normal level and moderately reduced the level of ALT (*P* < 0.01; Figure 3C), while no

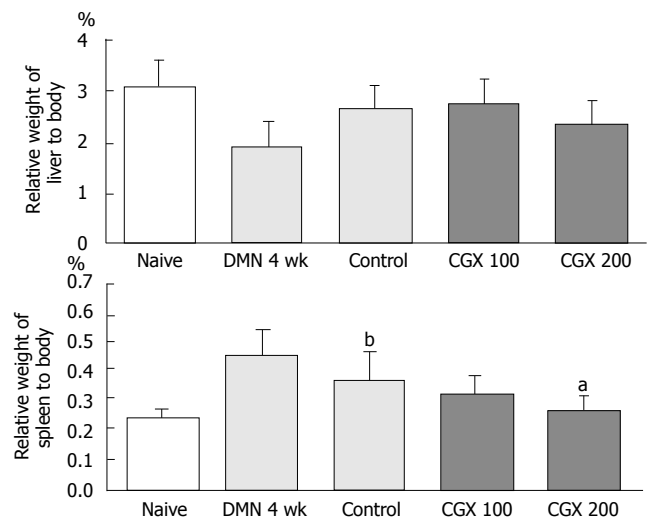


Figure 2 Weight of the liver and spleen relative to total body weight. Eight male Wistar rats per group were treated with DMN together with or without CGX as described in Figure 1C. At the end of the total treatment period, the liver and the spleen were removed from the rats and the relative organ weight with respect to total body weight was examined. ^a*P* < 0.05 vs control group; ^b*P* < 0.01 vs naïve group.

significant changes were observed after 2-wk cessation of DMN following the 4-wk DMN treatment without CGX.

Lipid peroxide and hydroxyproline

To examine the effects of CGX on lipid peroxidation and fibrosis in liver tissue, lipid peroxide and hydroxyproline were determined from frozen tissues. Prominent lipid peroxidation occurred during the 4-wk DMN treatment and progressed further during the 2-wk cessation period (control group), but 200 mg/kg CGX administration lowered it significantly (*P* < 0.01). Hydroxyproline, however, rapidly increased with DMN treatment but then dropped dramatically 2 wk later (control group), and CGX administration (100 and 200 mg/kg) significantly augmented its concentration (*P* < 0.01; Figure 4).

Histopathological findings

Four-week DMN treatment (DMN 4 wk) appeared to shrink the liver and cause it to become blunt and congested with blood, whereas the CGX treatment reversed these changes. These gross findings were supported by microscopic examination after hematoxylin & eosin and Masson's trichrome staining. DMN treatment for 4 wk caused severe local necrosis, inflammatory cell infiltration, hemorrhagic regions, and serious septal fibrosis. Although these pathologic features partially recovered by stopping the DMN treatment for 2 wk, CGX administration led to notable recovery effects (Figure 5).

Fibrosis-related gene expression analysis

To inspect the changes in gene expression of cytokines linked to liver fibrosis and the anti-fibrotic effects of CGX, gene expressions of TNF- α , TGF- β , PDGF- β , MMP-2, TIMP-1, and TIMP-2 were analyzed using real-time PCR. As shown in Figure 6, DMN treatment induced high expression of all these genes compared to the naïve group.

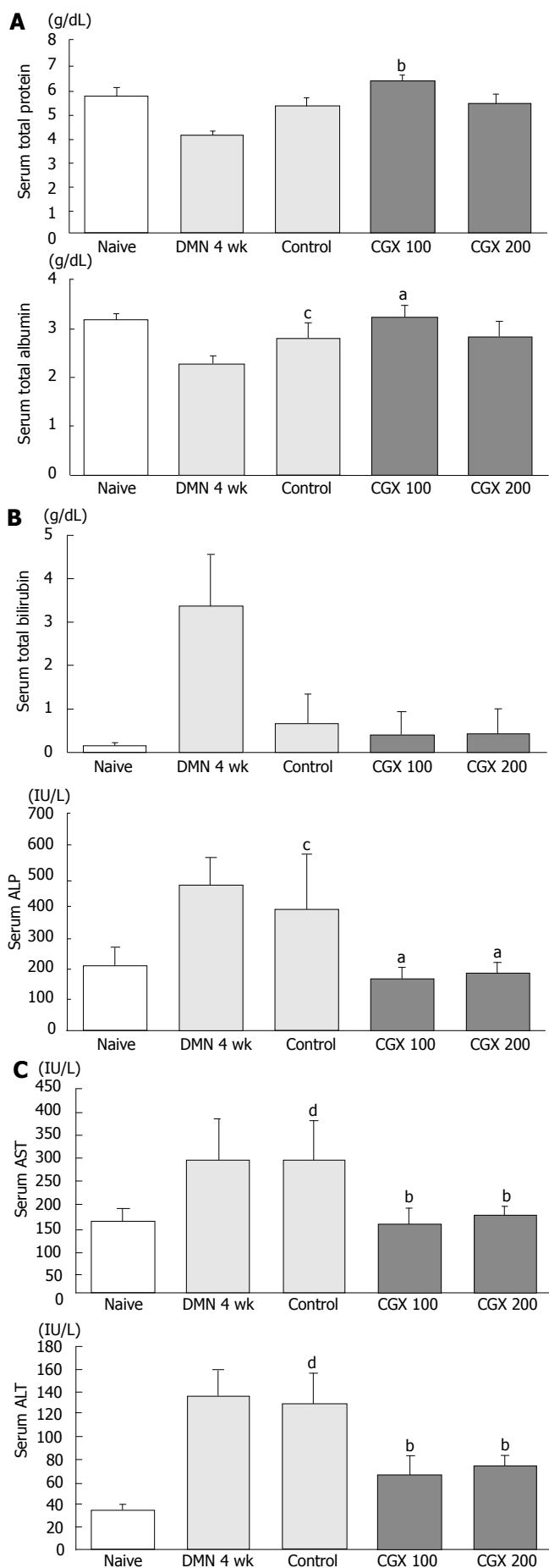


Figure 3 Serum biochemical analyses. At the end of the total treatment period, sera were prepared from blood collected via abdominal aorta of ether-anesthetized rats to measure (A) total protein and albumin, (B) total bilirubin and ALP, and (C) AST and ALT. ^a $P < 0.05$, ^b $P < 0.01$ vs control group; ^c $P < 0.05$, ^d $P < 0.01$ vs naive group.

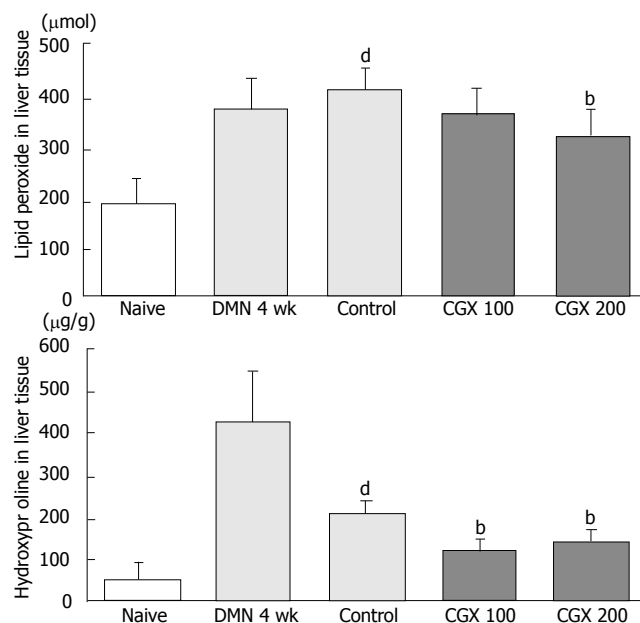


Figure 4 Measurement of lipid peroxide and hydroxyproline levels in liver tissues. Eight male Wistar rats per group were treated with DMN together with or without CGX as described in Figure 1C. At the end of the total treatment period, malondialdehyde and hydroxyproline were determined in the liver tissues. ^b $P < 0.01$ vs control group; ^d $P < 0.01$ vs naive group.

The DMN-induced high expression of $\text{TNF-}\alpha$, $\text{TGF-}\beta$, TIMP-1 , and TIMP-2 were spontaneously reduced to basal levels by cessation of the DMN treatment. However, the expression of genes encoding $\text{PDGF-}\beta$ and MMP-2 was further activated during the 2-wk cessation period (control group). These patterns of over-expressed genes prominently subsided following CGX administration.

DISCUSSION

Liver tissue is generally characterized by its vigorous ability to completely regenerate when damaged during short periods, whereas chronic liver injury can cause gradual progression of fibrotic or cirrhotic changes or sometimes cancerous transformation, resulting in dysfunction of the liver itself with whole-body consequences^[14]. Disorders with persistent destruction of liver tissue, known as chronic liver diseases, include chronic types of viral or alcoholic hepatitis, autoimmune hepatitis, liver cirrhosis, and liver cancer, and are a leading cause of death worldwide^[15]. The development of cirrhosis is a critical step impacting the clinical outcome or mortality of patients suffering from chronic liver diseases. Because cirrhotic changes consisting of fibrosis and nodulation are often a consequence of the wound-healing response to chronic liver injury, treating the underlying pathogenic conditions, such as chronic inflammation, before the onset of fibrogenesis is an important strategy for the management of these disorders^[16,17].

Despite the lack of well-designed clinical evidence, herbal medicines are becoming popular among patients with liver disease, and are attractive as putative anti-fibrotics or hepatotherapeutics^[18-22]. CGX has shown some hepatoprotective properties or anti-hyperlipidemic effects in previous studies^[9-11], so we further examined whether

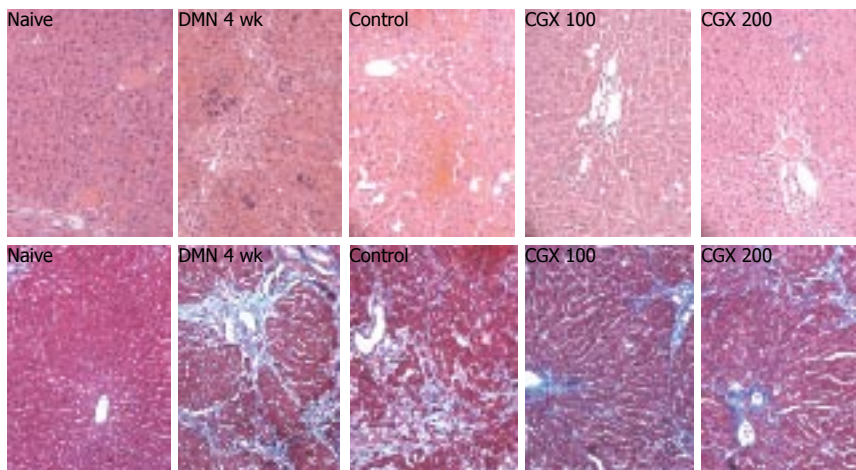


Figure 5 Histopathologic examination of liver tissues. Eight male Wistar rats per group were treated with DMN together with or without CGX as described in Figure 1C. At the end of the total treatment period, the liver was removed, fixed in Bouin's solution, and stained with hematoxylin and eosin (top, x 200 magnification) or Masson's trichrome (bottom, x 200 magnification) for histological examination under a light microscope.

this drug has therapeutic effects in a chronic liver damage model, including anti-fibrotic effects.

We constructed a chronic liver injury model using Wistar rats as in previous studies^[23-25], showing marked liver dysfunctions, such as low serum albumin, high level of bilirubin, ALP, AST, and ALT, and increased lipid peroxidation and hydroxyproline accumulation in liver tissues. In our experiments, the values of relative organ weights, serum albumin and bilirubin, and hydroxyproline in liver tissues moderately recovered to normal levels after 2-wk cessation of DMN without any treatment, whereas the levels of ALP, AST, and ALT remained dangerously out of normal physiologic conditions.

CGX administration showed major reducing effects on the increased levels of ALP, AST, and ALT induced by DMN treatment, and slight restorative effects on organ weight, serum albumin and bilirubin, and hydroxyproline concentration in liver tissues. In contrast to other biomarkers, the production of lipid peroxidation, measured as malondialdehyde (MDA), accelerated continuously up to the endpoint of this experiment in spite of stopping DMN treatment in the control group. This was partially restored by the administration of CGX (Figure 4). Because oxidative stress in DMN-treated animal models is known to be a major cause of hepatocellular damage and fibrosis^[26], it is likely that CGX has some hepatotherapeutic effects as a result of reducing DMN-induced oxidative stress.

As expected, DMN treatment for 4 wk resulted in the development of fibrosis, thus causing shrunken livers and the accumulation of hydroxyproline and extracellular matrix. Any chronic types of hepatocellular injury generally lead to hepatic fibrosis and cirrhosis, and investigation of anti-fibrotics and anti-cirrhotics have been a central issue due to their importance in the clinical outcome of patients. Thus far, no effective anti-fibrotic or anti-cirrhotic treatments are available for clinical use^[2,27]. This experiment demonstrated some anti-fibrotic effects of CGX administration, as shown by reduced hydroxyproline concentration in tissues and histological examination (Figure 5).

In order to determine the molecular mechanisms of these effects, we examined the change in expression of several fibrosis-associated genes in liver tissues. It has

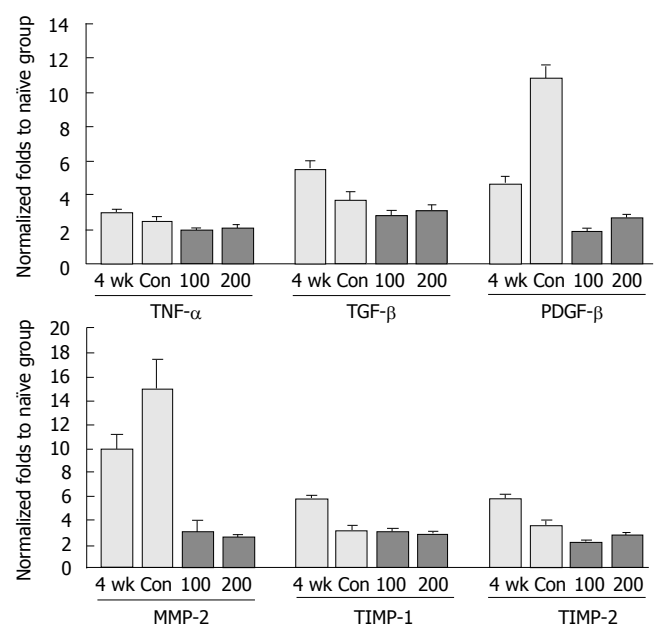


Figure 6 Expression of fibrosis-related genes using real-time PCR. Eight male Wistar rats per group were treated with DMN together with or without CGX as described in Figure 1C. At the end of the total treatment period, total RNA was prepared from liver tissues and used for cDNA synthesis. Quantitative real-time PCR was then performed using SYBR green supermix reagent with the gene-specific primers. The results are expressed in terms of fold increase in the expression levels relative to the naive control group. Con: Control.

already been established that stellate cells and various cytokines work as key factors in the pathogenic process of liver fibrosis^[2]. Among these, TGF-β and PDGF are considered the prominent profibrogenic cytokines^[28-31]. As shown in Figure 6, CGX administration moderately inhibited TGF-β expression compared to four- and five-fold over-expression in the DMN 4 wk and control groups, respectively. In particular, PDGF-β was obviously over-expressed by DMN treatment, whereas it was markedly inhibited by CGX administration. In addition, the unbalance between synthesis and degradation of the extracellular matrix is an important feature of liver fibrosis, and increased expression of several matrix metalloproteinases, including MMP-2 and tissue inhibitors of metalloproteinases (TIMPs), is well-known to occur during fibrogenesis^[32,33]. CGX administration also

noticeably decreased the expression of MMP-2 that had been highly over-expressed during or after DMN treatment, similar to previous reports^[34].

Among the many drugs derived from herbal medicines, silymarin is the most clinically popular for patients with various liver disorders, and is known to have hepatotherapeutic and anti-fibrotic properties^[35-37]. We also used silymarin (50 mg/kg) as a positive control in this experiment, and observed that CGX exhibited greater anti-fibrosis effects than silymarin (data not shown).

Based on these results, we conclude that CGX possesses hepatotherapeutic properties against chronic hepatocellular destruction and consequential liver fibrosis. The mechanism of these therapeutic effects of CGX on liver diseases, however, should be further clarified so that CGX can be used as effectively as possible as a means of treatment.

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Hydrodynamics based transfection in normal and fibrotic rats

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following HBT results in the formation of large endothelial gaps. These gaps, though important in the transfer of DNA molecules from the blood to the space of Disse are not enough to provide the appropriate conditions for hepatocyte transfection. Hydrodynamics based injection is applicable in fibrotic rats provided that ECM load is reduced.

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Key words: Gene transfection; Fibrosis; *In vivo* transfection; Fibrotic; Hepatic; Endothelial lining; Endothelium; Sinusoidal; Fenestrae; Space of disse; Extracellular matrix

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Abstract

AIM: Hydrodynamics based transfection (HBT), the injection of a large volume of naked plasmid DNA in a short time is a relatively simple, efficient and safe method for *in vivo* transfection of liver cells. Though used for quite some time, the mechanism of gene transfection has not yet been elucidated.

METHODS: A luciferase encoding plasmid was injected using the hydrodynamics based procedure into normal and thioacetamide-induced fibrotic Sprague Dawley rats. Scanning and transmission electron microscopy images were taken. The consequence of a dual injection of Ringer solution and luciferase pDNA was followed. Halofuginone, an anti collagen type I inhibitor was used to reduce ECM load in fibrotic rats prior to the hydrodynamic injection.

RESULTS: Large endothelial gaps formed as soon as 10' following hydrodynamic injection; these gradually returned to normal 10 d post injection. Hydrodynamic administration of Ringer 10 or 30 m prior to moderate injection of plasmid did not result in efficient transfection suggesting that endothelial gaps by themselves are not sufficient for gene expression. Gene transfection following hydrodynamic injection in thioacetamide induced fibrotic rats was diminished coinciding with the level of fibrosis. Halofuginone, a specific collagen type I inhibitor, alleviated this effect.

CONCLUSION: The hydrodynamic pressure formed

INTRODUCTION

The ability to introduce foreign genes has paved the way for elucidating the physiological functions of genes and their therapeutic potential in the context of the entire organism^[1,2]. A crucial yet unresolved issue in successfully implementing gene delivery as an effective procedure is the methodology by which genes can be introduced. In recent years naked DNA rather than synthetic vectors or viruses were applied to animal models. In doing so, various procedures were used including direct injection to liver vessels or bile duct^[3-7].

The introduction of the hydrodynamics based transfection (HBT) by Liu *et al*^[2] and Zhang *et al*^[1] presented new opportunities in the field owing to its relatively straightforward manner, eliminating the need for surgical procedures. The technique has been widely used ever since, reaching beyond the goals of treating liver diseases. Gene suppression by RNAi or defining regulatory DNA sequences^[8,9] are among the topics investigated using this approach. Two parameters influence more than any other the outcome of the hydrodynamic injection, the volume administered and the rate of injection. In the mouse model up to 10% of body weight is administered within 5 s. Under these harsh conditions the output of the heart fails to cope with the heavy load of fluid, leading to excessive blood flow into the hepatic vein circulation and consequent transfection of hepatocytes^[10].

Attempts to elucidate the mechanism underlying the HBT have yielded conflicting reports. These reports

include receptor mediated endocytosis^[11], non specific entry to hepatocytes through transient small pores caused by high pressure^[12] or transient inversion of intrahepatic flow associated with massive endocytosis^[10]. Regardless of the mechanism, it is obvious that the sinusoids and associated cells, i.e., sinusoidal endothelial cells, hepatic stellate cells and Kupffer cells are influenced by the high pressure created by the procedure.

The current study follows the fate of the reporter gene luciferase in fibrotic rats undergoing HBT. Such animals demonstrated lower levels of transfection compared to healthy control rats, yet the outcome of the HBT could be improved by halofuginone, an anti collagen type I inhibitor. We also bring evidence demonstrating that the morphological alterations in the form of fused fenestrae, gaps, inflicted on the membrane of sinusoidal endothelial cells following HBT are either not involved or not sufficient to achieve transfection of liver cells.

MATERIALS AND METHODS

Plasmid

pGL3-Control vector (Promega, Madison, WI) encoding for the luciferase gene was purified using a commercial kit (Qiagen, Valencia, CA). The purity of the plasmid was checked by Nanodrop (Wilmington, DE) and agarose gel electrophoresis.

Hydrodynamic injection and thioacetamide administration

Sprague-Dawley rats (250-280g) were injected via the tail vein with luciferase encoding plasmid using a 22G venflon cannula in a volume equivalent to 5.25% of the body weight and a rate of 5-8 s. The amount of DNA administered throughout this study, 400 µg/injection, was deduced from a preliminary study in which various amounts of naked DNA were used. In all experiments the DNA was dissolved in Ringer solution (117 mmol/L NaCl, 2.5 mmol/L KCl, 2 mmol/L CaCl₂, 9.1mmol/L Hepes, 5 mmol/L D-glucose pH 7.4). Prior to injection rats were anesthetized using Ketamin HCl (90 mg/kg) and Acepromazine Maleate PB (2.5 mg/kg). The amount of Acepromazine Maleate PB was reduced to 1 mg/kg in fibrotic rats. Liver fibrosis was induced by injection of 0.2 mg/g thioacetamide (TAA) intraperitoneally twice weekly for the designated time. The level of fibrosis was determined using the Ishak scoring system^[13] following staining with Sirius Red. Halofuginone (Collgard, Petach Tikva, Israel) was supplied in the food at a concentration of 5 ppm.

Histochemistry

Liver samples were fixed in 4% paraformaldehyde, dehydrated in gradual ethanol solutions and embedded in paraffin. Five µm thick sections were blocked for endogenous peroxidase and stained with Sirius Red^[14].

Luciferase assay

Two hundred mg of liver tissue were homogenized by Polytron homogenizer (Kinematika, Lucerne, Switzerland) in 1ml Reporter lysis buffer (Promega Corp., Madison, WI). Luciferase activity was determined using a commercial assay kit (Bright-Glo Luciferase Assay System, Promega,

Madison, WI) according to manufacturer's instructions. Luciferase was expressed as Relative Light Units per mg protein ± standard deviation.

Cell proliferation

BrdU labeling was used to determine cell proliferation. Briefly animals were intraperitoneally injected one and two hours before sacrifice with PBS containing 5-bromo-2-deoxyuridine (Sigma, St. Louis, MO) at a dose of 50 mg/kg body weight. Liver samples were fixed, dehydrated, embedded in paraffin and cut to 5 µm slices. Staining was performed using the BrdU kit (Zymed, South San Francisco, CA).

Electron microscopy

Liver tissue samples were prepared according to standard protocols^[15]. Briefly, liver tissue was prefixed, cut to 1 mm³ samples and stored in 1.5% glutaraldehyde in 0.12 mol/L cacodylate buffer at pH 6.9. Following glutaraldehyde fixation, tissue blocks were postfixed in 1% Osmium tetroxide. Dehydrated tissue blocks were dried with hexamethyldisilazane and subsequently broken in liquid nitrogen, mounted on stubs and sputter coated with a thin layer of 20 nm gold^[16]. Scanning electron microscopy (SEM) samples were studied in a Philips XL-20 at 30 kV. Morphometric analysis was performed on randomly acquired digitized SEM images at magnifications × 5000 or × 20000 as previously described^[17]. The UTHSCSA Image Tool 2.0 software was used to trace the number and diameter of liver sinusoidal endothelial gaps. Gaps were discriminated from fenestrae based on their morphology and size as described^[15,16,18]. Each experimental variable was repeated three times (3 animals) using 10 images in the periportal and pericentral zone (regions up to 100 µm in diameter) randomly selected and captured at both magnifications. All experiments were repeated three times and data were expressed as mean ± standard deviation of the mean.

Statistical analysis

Statistical analysis was performed with the Mann Whitney two-sided U-test for endothelial gap sizes and one way ANOVA for plasmid distribution in the liver lobes. The t-test was used for plasmid expression in the different experimental groups.

RESULTS

Luciferase gene distribution in rat liver lobes following HBT

Given the conflicting reports as to the distribution of naked DNA in the liver following HBT^[19-21] we first addressed the above, using the pGL3-Control vector. Following HBT, random segments from each of the four liver lobes were thus picked, minced in Reporter lysis buffer (Promega, Madison, WI) supplemented with Complete Mini Protease Inhibitor (Roche Diagnostics, Mannheim, Germany) and tested for luciferase. Surprisingly, variations in luciferase expression were noted between segments of the same lobe. To avoid false interpretation in all further experiments, liver lobes were chopped into small pieces and a 200 mg representative sample was collected and

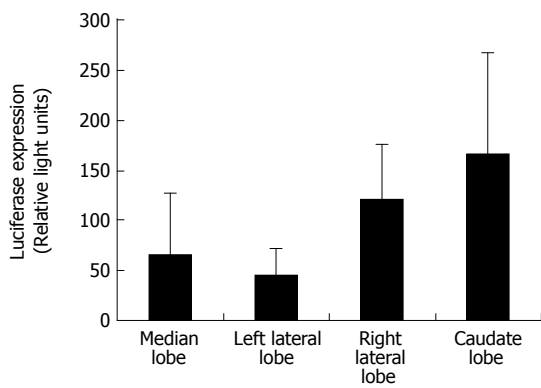


Figure 1 400 μ g of pGL3-Control plasmid DNA was injected in 5.25% (volume per animal weight) Ringer to healthy rats by the hydrodynamics based injection (5 to 8 s). 24 h later the liver was excised, the lobes minced and 200 mg samples of each of the four lobes homogenized in reporter lysis buffer and luciferase activity monitored ($n = 10$).

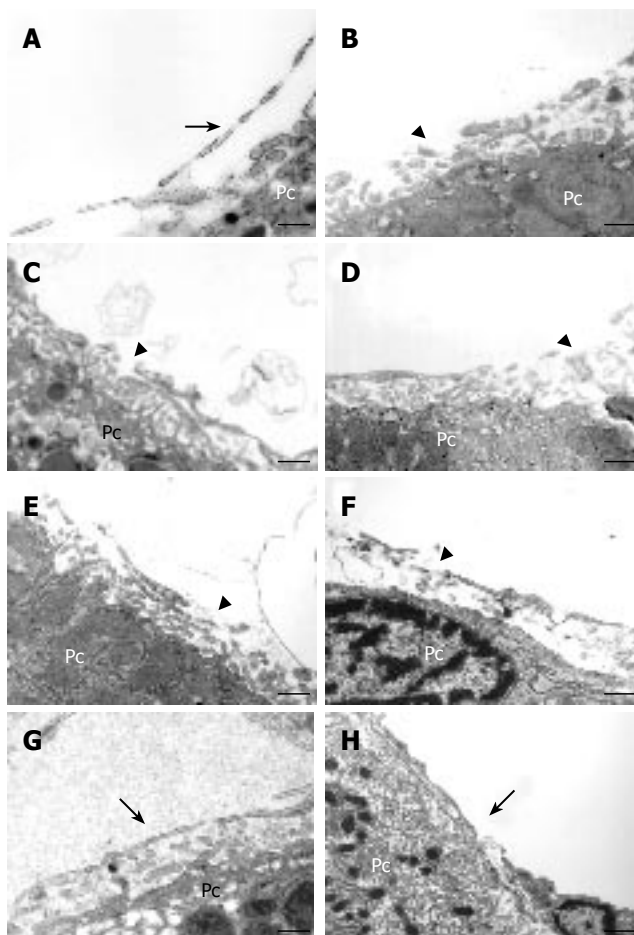


Figure 2 High magnification electron micrographs of liver sinusoids following hydrodynamic injection. **A:** Control image illustrates intact histological relationship between liver sinusoidal endothelial and neighboring liver parenchymal cells (Pc). Note the fenestrated processes (arrow) of endothelial cells; **B:** Ten minutes after hydrodynamic injections, severe damage of the endothelial lining in the form of gaps is noted (arrowhead). Similar features were observed at 1 (**C**), 8 (**D**), 24 (**E**), and 72 (**F**) h, i.e., a disrupted endothelial lining with the presence of large structural rearrangement in the form of gaps (arrowheads). The architecture of hepatocytes remained intact when compared to the control. Between seven (**G**) to ten (**H**) days post injection, the number of gaps decreased significantly. Interestingly, the hepatic sinusoidal endothelial lining is less fenestrated when compared to the control (arrow). Scale bars, 1 μ m.

Table 1 Number of gaps along the sinusoidal endothelial lining after hydrodynamic injections (mean \pm SD, $n = 30$)

	<i>n</i> gaps/10 μ m ² (periportal)	<i>n</i> gaps/10 μ m ² (pericentral)
Control	0.14 \pm 0.15	0.18 \pm 0.21
10 min	3.04 \pm 0.62 ^{a,c}	0.86 \pm 0.42 ^e
1 h	3.02 \pm 0.35 ^{a,c}	0.76 \pm 0.42 ^e
8 h	3.08 \pm 0.64 ^{a,c}	1.39 \pm 0.46 ^e
24 h	2.98 \pm 0.55 ^{a,c}	1.07 \pm 0.39 ^e
72 h	1.42 \pm 0.32 ^{a,c}	0.72 \pm 0.27 ^e
168 h	0.92 \pm 0.33 ^{a,c}	0.49 \pm 0.21
240 h	0.45 \pm 0.19	0.39 \pm 0.20

Scanning electron microscopy based morphometric analysis of gaps per area along the sinusoidal endothelial lining cells. Significance of data was determined using the Mann Whitney two-sided U-test. Significant differences between the number of gaps in the periportal and pericentral zones were noted at all time points indicated ($^*P \leq 0.05$) following hydrodynamic injections. Significant differences between control and time points indicated following hydrodynamic injections were evident at both periportal ($^*P \leq 0.05$) and pericentral ($^*P \leq 0.05$) areas of the liver. A gap is defined as a hole with a diameter size of 300-2000 nm.

used to give a reliable representation of the expression in the lobe (Figure 1). Comparison between the four lobes showed statistical difference in expression (d.f. = 3, $F = 6.78$, $P < 0.001$). The left lateral lobe was found by post hoc analysis to express significantly less plasmid ($P < 0.05$).

HBT is associated with the formation of large endothelial gaps

An enlargement in liver fenestrae was reported when high pressure was used in liver perfusion^[22]. We have demonstrated an increase in blood flow to the liver following partial hepatectomy which was associated with the formation of "fused" fenestrae (gaps)^[23]. The number of these gaps gradually decreased, reaching baseline level 10 d post surgery. To test the effect of hydrodynamic injections on cells occupying the space of Disse, both scanning and transmission electron microscopy were utilized. Prior to HBT injection, normal ultrastructure of liver sinusoids and the surrounding hepatocytes was noted, with patent lumen delineated by endothelial cells with thin processes containing a few fenestrae. These fenestrae ranged in size between 120-140 nm. Microvilli extending from the parenchymal cell surface could be readily seen. Hepatocytes show normal ultrastructure displaying a cytoplasm rich in rough endoplasmic reticulum, glycogen and multiple membrane bound vesiculo-organelles such as lysosomes, lipid droplets and coated pits. As early as ten minutes after hydrodynamic injections, endothelial-associated changes were noted in the form of fused fenestrae, i.e., gaps, ranging in diameter between 0.3 μ m and 2 μ m. These gaps were more prominent in periportal areas than pericentral areas (Figure 2 and Table 1). Increasing values were noted in subsequent times, i.e., 1 to 24 h post-hydrodynamic injection in both areas. The number of gaps decreased gradually reaching almost pre-hydrodynamic injection levels 240 h post injection (Table 1). Scanning electron microscopy revealed similar results (Figure 3). Control tissue demonstrated intact relationship between sinusoidal endothelial cells and neighboring liver parenchymal cells. Ten minutes after injections the

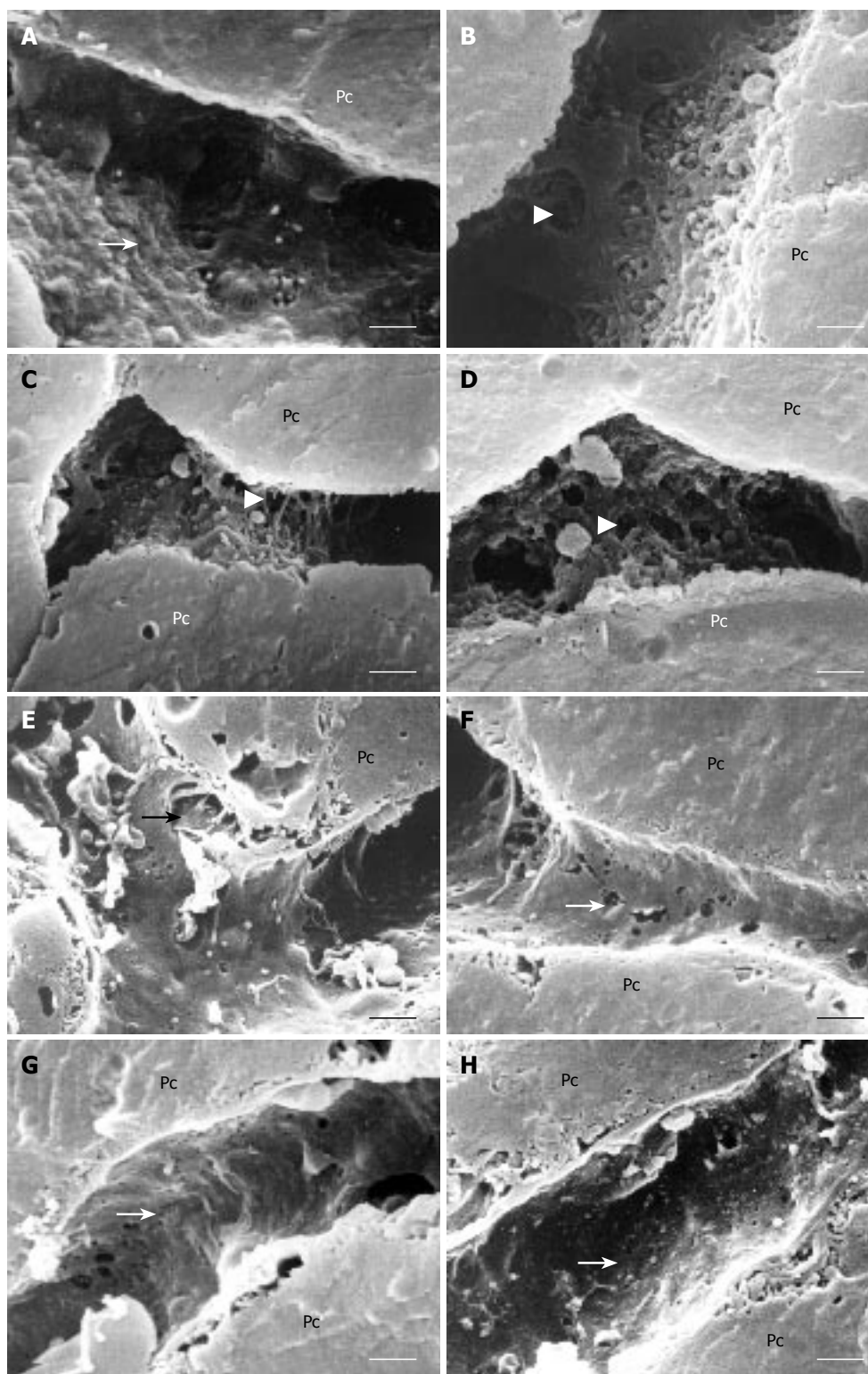


Figure 3 Scanning electron micrographs of liver sinusoids following hydrodynamic injections. **A:** Control liver demonstrates an intact endothelial wall with numerous small pores perforating the endothelial lining (fenestrae) (arrow) and undisrupted bordering parenchymal cells (Pc); **B:** Numerous gaps (arrowhead) are observed as early as ten minutes after hydrodynamic injections and persist at one (**C**) and eight (**D**) hours after injection. From twenty four hours post HBT and onwards the number of gaps (arrowhead) decreases, yet a significant number are still present (**E**, **F**). Between seven (**G**) and ten (**H**) days following HBT, the endothelial lining reveals similar features as described under Figure 2G-H: i.e., less fenestrated appearance. Scale bars, 2 μ m.

endothelial lining was disrupted by gaps and microvilli facing toward the sinusoidal lumen. Ten days after HBT liver morphology returned to normal with very few fenestrae.

No increase in BrdU cell labeling was evident in HBT injected rats (data not shown) suggesting that in spite of the morphological damage mentioned above no cell proliferation was associated with our protocol.

Hydrodynamic administration of Ringer solution prior to transfection does not facilitate plasmid transfection

Given the fact that endothelial cell fenestrae undergo major

changes resulting in a significant increase in the number of gaps as early as 10 m following HBT we examined whether the induction of gaps by itself enables plasmid DNA transfection. Normal rats were thus injected with Ringer solution in a volume equal to 5.25% of their body weight followed by a moderate injection of 400 μ g of pGL3-Control plasmid 10 or 30 m post HBT. Two control groups of rats were injected either hydrodynamically or moderately with the same plasmid. While hydrodynamic administration of the plasmid resulted in a remarkably high level of luciferase expression, hydrodynamic administration of Ringer followed by moderate injection

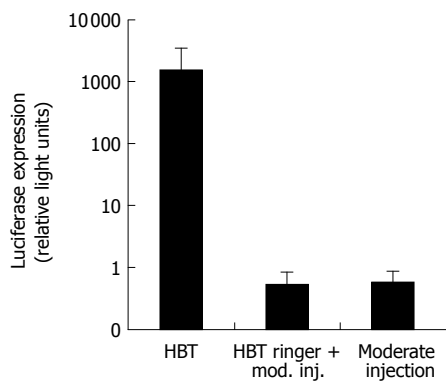


Figure 4 400 μ g of pGL3-Control plasmid DNA were injected in the following variations: (1) HBT: 5.25% (volume per animal weight) Ringer solution with plasmid DNA in 5 to 8 s; (2) HBT Ringer + moderate injection: 5.25% (volume per animal weight) Ringer solution in 5 to 8 s followed by 400 μ g of plasmid DNA in 500 μ L 10 or 30 m later; (3) moderate injection: 500 μ L Ringer solution with 400 μ g plasmid DNA. Twenty four hours post injection the liver was excised. 200 mg liver sample were then homogenized in reporter buffer and luciferase activity monitored ($n = 5$).

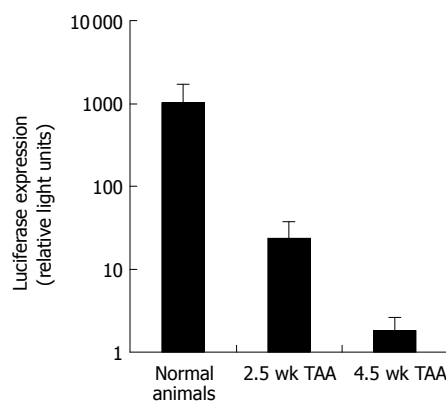


Figure 5 400 μ g of pGL3-Control plasmid DNA was injected in 5.25% (volume per animal weight) Ringer in 5-8 s to normal rats, and rats injected intraperitoneally (IP) twice weekly with thioacetamide (TAA) for either 2.5 or 4.5 wk. 24 h later the liver was excised and 200 mg samples of the median lobe were homogenized in reporter lysis buffer and the activity of luciferase monitored ($n = 5$).

of the plasmid 10 or 30 m after HBT yielded significantly lower expression levels ($P < 0.05$), similar to those of moderate injection ($P > 0.5$ between these two groups) (Figure 4).

Transfection efficiency of HBT in fibrotic rats is reduced

HBT is associated with an increase of hydrodynamic pressure in the liver, leading to an efficient transfection. It follows therefore that “blocking” endothelial sinusoids would lead to gradual decrease in the efficiency of transfection. To test this assumption we followed the fate of pGL3-Control in rats treated with thioacetamide for 2.5 or 4.5 wk. Such treatment results in fibrotic expansion of most portal areas along with the formation of incomplete septa (Ishak scoring 1-2) and portal to portal and portal to central septa including occasional nodules (Ishak scoring 5) respectively. HBT in both fibrotic groups resulted in a significant reduction in the expression of luciferase ($P < 0.000$). Between the two fibrotic groups there was also significant difference in expression ($P < 0.005$) (Figure 5). Furthermore, luciferase expression strongly correlated

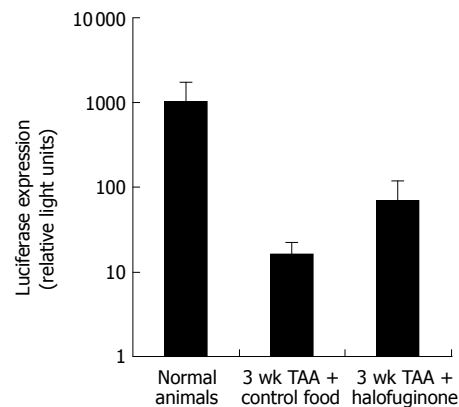


Figure 6 400 μ g of pGL3-Control plasmid was injected in 5.25% (volume per animal weight) Ringer in 5-8 s to normal rats, and rats previously injected intraperitoneally twice weekly with thioacetamide (TAA) for 3 wk. Prior to the hydrodynamic injection the rats were fed for 3 wk with either control food or food containing halofuginone at a concentration of 5 ppm. Twenty four hours post HBT the liver was excised and 200 mg samples of the median lobe were homogenized in reporter lysis buffer and the activity of luciferase monitored ($n = 5$).

inversely with the severity of fibrosis as quantitated by Ishak ($r = -0.77$). Halofuginone is a specific collagen type I inhibitor. In previous studies^[24,25] we have shown its beneficial usage in reducing collagen deposition in fibrotic rats. When given to rats with established fibrosis prior to hydrodynamic injection, an increase in the level of luciferase expression following HBT was evident ($P < 0.05$ between the treated and untreated groups) (Figure 6). Such animals also demonstrated a reduced level of fibrosis as determined by Sirius Red staining.

DISCUSSION

Hydrodynamics based transfection allows efficient delivery of plasmid DNA to the liver. This simple procedure has been used to test the potential benefits of certain genes like the hemophilia Factor IX or for screening novel elements *in vivo*^[26-28]. Yet in spite of the intense usage of the methodology, the mechanism underlying the entry and expression of the targeted gene is still unclear. The present study elucidates basic features associated with the technique, which are of fundamental importance when the technique is being implemented. It also demonstrates the potential usage of halofuginone, a collagen type I inhibitor in the event that the HBT technology is used in fibrotic rats. Conflicting reports exist as to the number of cells expressing the desired gene following HBT. Song and colleagues identified close to 40% positively stained cells most of which are hepatocytes^[29]. Others^[3] reported significantly lower numbers of 10% or less. It is obvious that incorrect sampling of the liver may lead to a wrong conclusion. Our current analysis using samples representing the complete lobe previously cut into small pieces was reliable and reproducible. In that context, it is worth noting that variations in gene expression between the four liver lobes were noted, though these differences were not significant except for the left lateral lobe which showed lower expression ($P < 0.05$). We conducted all experiments using the median lobe which is the largest.

The term “liver sieve” is used to describe the endothelial

cells that line the hepatic sinusoids. These fenestrated endothelial cells which have no basement membrane allow free transfer from the sinusoidal lumen of various substrates including growth factors, hormones, proteases and chylomicrons^[15]. Evidence points to the availability of receptors responsible for the removal of soluble molecules via sinusoidal endothelial cells (SEC)^[30]. Fenestration of sinusoidal endothelial cells is a dynamic process whose size and density may be affected by various factors ranging from physical factors to soluble substances^[31]. The formation of large gaps following an increase in hydrodynamic pressure in the liver has been documented in different set-ups. Fraser and colleagues have shown enlarged fenestrae when high pressure was applied in a liver perfusion model^[22]. Wack and colleagues^[31] reported increased porosity and fenestration diameters following 70% partial hepatectomy. We have recently been able to demonstrate the formation of large gaps ranging in diameter between 0.3 μm and 2 μm following 70% partial hepatectomy^[23]. These gaps are most probably formed by the increased shear stress associated with elevated blood flow following hepatectomy. Though gaps are evident 10 m post hepatectomy, it is likely that these are formed concomitantly with the elevation in blood flow. We now show a similar pattern of gap formation following hydrodynamic injection. In both cases, the increase in the number of gaps was more prominent in periportal areas than pericentral areas. These differences cannot be simply explained by higher pressure formed close to portal zones as increased pressure in the inferior cava vein and therefore in central veins has been described with the hydrodynamic injections^[10]. The reported increase in the number of gaps was evident 10 m post HBT, peaking between 8 and 24 h. Gradual recovery was thereafter recorded approaching normal values ten days post injection.

For naked pDNA to be expressed following HBT it needs to overcome the obstacle of the lining endothelial cells, and reach the hepatocytes which constitute the majority of cells transfected under hydrodynamic pressure, penetrate the membrane and arrive in the nucleus. The formation of gaps, sized up to 0.3 μm following HBT seems to facilitate the crossing of DNA from the blood to the space of Disse. Further, such gaps may also help clear pDNA from the blood instantaneously, thereby avoiding exposure to nucleases and rapid degradation^[12,32]. The importance of the space of Disse and the state of its ECM components on the expression of naked pDNA using the hydrodynamic technology was proven in rats previously administered with thioacetamide.

HBT in fibrotic rats was less effective compared to healthy control rats. Further, the expression of luciferase correlated with the level of fibrosis. Liver fibrosis is associated with both qualitative and quantitative changes in the composition of hepatic ECM. In the healthy liver the endothelium expresses very little collagen thus minimizing the barrier to substrate diffusion^[30,33]. Following injury, the total content of ECM increases 3-5 times and the sub-endothelial type of ECM shifts from normal low density basement membrane like matrix to interstitial type matrix containing fibril forming collagen^[34]. These changes have been reported to also be associated with

the closure of HSC fenestrae^[35]. The low expression of luciferase achieved in fibrotic rats undergoing HBT thus seems to coincide with the deposition of ECM and the blocking of the fenestrae. Given the fact that collagen type I may account for 5%-30% of the deposited ECM components in fibrotic liver, drugs inhibiting collagen synthesis or supporting collagen degradation should be used to help gene expression following HBT. In the present study we used halofuginone to reduce ECM content induced by thioacetamide. Halofuginone is a well known collagen I inhibitor. The drug has been proven effective in various models^[36-40] including fibrotic rats using either dimethylnitrosamine or thioacetamide^[25,40]. In rats exhibiting moderate levels of fibrosis halofuginone treatment resulted in a decrease in the level of fibrosis. The expression of luciferase in this group was higher than the control group, demonstrating its efficacy.

The mechanism underlying the HBT technology is still unresolved. The formation of endothelial gaps associated with the hydrodynamic pressure developing in the liver following HBT may account for the transfer of pDNA from the blood to the space of Disse, yet it does not explain why liver cells, mainly hepatocytes, are transfected by the same pDNA. Indeed when a moderate injection of pGL3-Control (500 μL) was performed 10 or 30 m following hydrodynamic injection of Ringer solution, the level of luciferase expression was the same as that obtained following moderate injection only. The increase in the number of endothelial gaps as early as 10 m post injection suggests that they are either not involved in the transfection procedure or are not the only event occurring following HBT.

In a separate study^[19,41] an interval longer than 10 s between the two injections results in a significant and gradual decrease in the number of cells expressing the desired gene.

Isolated hepatocytes shortly after HBT demonstrate maximal pDNA expression, suggesting that DNA molecules have been taken up soon after injection. While our data on the formation of gaps generally coincide with that reported by others^[32] we found no evidence for the formation of membrane pores following HBT. If indeed DNA molecules enter hepatocytes through pores, then a certain concentration of DNA should be achieved within a short time and the number of pores should be sufficient to allow entry. Based on experimental data and theoretical assumptions Budker and colleagues^[11] are not in favor of the membrane pore mechanism but rather support the involvement of a receptor mediated process. Regardless of the unresolved mechanism underlying the hydrodynamic injection our study paves the way for using the technology in fibrotic animals as well. The usage of halofuginone in partially resolving some of the accumulated ECM components in the liver should encourage the search for drugs that might be used in future combined therapy.

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BASIC RESEARCH

Role of vasoactive intestinal peptide and nitric oxide in the modulation of electroacupuncture on gastric motility in stressed rats

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Abstract

AIM: To investigate the effects and mechanisms of vasoactive intestinal peptide (VIP) and nitric oxide (NO) in the modulation of electroacupuncture (EA) on gastric motility in restrained-cold stressed rats.

METHODS: An animal model of gastric motility disorder was established by restrained-cold stress. Gastric myoelectric activities were recorded by electrogastroenterography (EGG). VIP and NO concentrations in plasma and gastric mucosal and bulb tissues were detected by radioimmunoassay (RIA). VIP expression in the gastric walls was assayed using avidin-biotin-peroxidase complex (ABC) and image analysis.

RESULTS: In cold restrained stressed rats, EGG was disordered and irregular. The frequency and amplitude of gastric motility were higher than that in control group ($P < 0.01$). VIP and NO contents of plasma, gastric mucosal and bulb tissues were obviously decreased ($P < 0.01$). Following EA at "Zusanli" (ST36), the frequency and amplitude of gastric motility were obviously lowered ($P < 0.01$), while the levels of VIP and NO in plasma, gastric mucosal and bulb tissues increased strikingly ($P < 0.01$, $P < 0.05$) and expression of VIP in antral smooth muscle was elevated significantly ($P < 0.01$) in comparison with those of model group.

CONCLUSION: VIP and NO participate in the modulatory effect of EA on gastric motility. EA at "Zusanli" acupoint (ST36) can improve gastric motility of the stressed rats by increasing the levels of VIP and NO.

INTRODUCTION

In recent years, along with the extensive research into enteric nerve system (ENS), increasing evidence shows that peptidergic neurotransmitters are the key factors regulating the gastric motility. Our previous research^[1-3] showed that electroacupuncture (EA) at acupoints of the Stomach Meridian of Foot-Yangmin may regulate gastric movement, increase blood flow in the microvessels in the gastric mucosa, and exert a protective effect on gastric mucosa. Nitric oxide (NO) and vasoactive intestinal peptide (VIP) participate in the protective effect of EA against gastric mucosal damage. It has been demonstrated that the increase or decrease of NO can lead to electrogastric dysrhythmias^[4]. Regular gastric motility in antrum was abolished after traumatic stress in rats. Plasma VIP contents were increased significantly within 2 h after stress^[5]. The restraint plus water-immersion stress in rats induced obvious enhancement in gastric motility. NO levels of gastric mucosa were decreased significantly, and the gastric mucosal lesion was obvious^[6]. VIP may be important in the prevention of gastric mucosal damage induced by cold-restraint stress^[7]. Therefore, the aim of this study was to investigate the modulative effect of EA on gastric motility and its relation to NO and VIP in the animal model of restrained-cold stress, in an effort to explore the mechanisms of EA.

MATERIALS AND METHODS

Animal treatment

Eighty Wistar rats (weighing 220 ± 30 g), provided by the Experimental Animal Center of Anhui Medical University, were randomly divided into normal control group, model

group, EA group and non-acupoint group, with 20 animals in each. Each group was further divided into two parts: 10 rats measured by radioimmunoassay, and another 10 rats measured by immunohistochemistry. Rats of the last 3 groups were subjected to 24 h of fasting (including water-intake), then anesthetized with 20% urethane (1.0 g/kg), and fastened to an animal board and put into a refrigerator at 4°C for 2 h after awaking. Bilateral “Zusanli” (ST36) were punctured with filiform needles and stimulated with an EA therapeutic apparatus and using parameters such as frequency of 20-100 Hz, dense-sparse waves, electrical current of 2-3 mA and duration of 30 min. The non-acupoint group was located at the site of the buttock on the bilateral sides. The treatment was given once daily, for 7 consecutive days.

Gastric myoelectric activity

Rats were anesthetized with 20% urethane (1.0 g/kg) and an Ag-AgCl electrode was fixed on the body surface at the projection spot of antrum (1.5 cm below xiphoid process, 0.5 cm leftward). The positive electrogastroenterography (EGG) electrode was connected with the lead column of the surface electrode and the negative electrode was connected with right lower limb, then connected to earth. Gastric myoelectric activities were measured by EGG^[8]. Frequency and amplitude of EGG were recorded and analyzed by computer^[9].

Measurement of VIP and NO concentrations

At the end of each experiment and after decapitation of the rat, 3 mL of blood sample was taken and put into a test tube containing 30 µL of 10% EDTA-Na₂ (an anticoagulant) and 40 µL of trasylol, and mixed evenly. The blood sample was refrigerated at 4°C-8°C for 15 min, and then centrifuged at 3500 r/min, 4°C for 5 min to separate the plasma, which was put into a 1 mL tube, stored in the refrigerator at -20°C. The gastric mucosal and bulb tissues were weighed and added to 0.5 mL of 1 mol/L acetic acid and mixed evenly in a homogenizer to obtain a homogenate. The extracted homogenate was added to 0.5 mL of 1 mol/L NaOH and centrifuged at 3000 r/min, 4°C for 30 min. The supernatant fluid was collected and stored at -20°C.

The concentrations of VIP in the plasma and supernatant were determined by radioimmunoassay. The gastric mucosal and bulb tissues were boiled in 2-3 mL of normal saline for 3 min. The NO contents in blood and mucosa of gastric antrum and bulb tissues were assayed according to the method by Green *et al.*^[10]. The test kit was supplied by Beijing Huaying Bio-technique Institute.

Immunocytochemistry and image analysis

The expression of VIP in gastric wall was assayed using avidin-biotin-peroxidase complex^[11]. Rats were anesthetized with intraperitoneal injection of sodium pentobarbital (30 mg/kg). Thoracotomy and aortic cannulation were performed. After flushing of the blood with saline, the rats were perfused iv rapidly with 400 mL of fixative (4% paraformaldehyde and 0.5% glutaraldehyde) and then with slow iv drip infusion for 1 h. Antral tissues were fixed in

Table 1 Effect of EA on gastric electroactivity of antrum under restrained-cold stress in rats (mean ± SD)

Groups	n	Frequency (cpm)	Amplitude (µv)
Control	20	3.07 ± 0.55 (0.18)	363.21 ± 42.41 (0.12)
Model	20	4.26 ± 0.58 ^b (0.14)	428.54 ± 56.23 ^b (0.13)
EA	20	3.41 ± 0.48 ^d (0.14)	378.62 ± 24.38 ^d (0.06)
Non-acupoint	20	4.12 ± 0.36 (0.09)	407.25 ± 65.42 (0.16)

^b*P* < 0.01 *vs* control group; ^d*P* < 0.01 *vs* model group. Numerical value in brackets is variation coefficient.

4% paraformaldehyde for 3 h and then immersed into 20% cane sugar fluid for 12 h at 4°C in a refrigerator. The frozen tissue was serially sectioned at 30 µm thickness and mounted onto glass slides. Changes in VIP distributions in antral smooth muscle were observed by immunocytochemistry and image analysis. VIP monoclonal antibody was purchased from Sigma Chemical, USA. SABC kit was purchased from Boshide Biotechnology Co, Wuhan. Detection was carried out according to the instruction of the kit. Five visual fields in three sections of each tissue, were randomly selected and observed under the light microscope and analyzed with LeicaQ500IW image analysis system for the integral optical density (IOD).

Statistical analysis

Data were expressed as mean ± SD, and analysed by SPSS 11.0 software. Analysis of variance and *t* test were used for intergroup comparison. *P* < 0.05 was considered statistically significant. *P* < 0.01 was considered statistically obviously significant.

RESULTS

Gastric myoelectric activity

EGG was regular with frequencies of 3.07 cycle per min (cpm) and amplitudes of 360-370 µv in the control group. In rats with gastric motility disorder induced by cold restraining stress, EGG was disordered and irregular. There was an obvious difference in frequency and amplitude of EGG between the model group and control group (*P* < 0.01). Following EA at “Zusanli” (ST36), the frequency and amplitude of gastric motility were obviously decreased (*P* < 0.01). In the non-acupoint group, the two parameters had no obvious difference compared with those of model group (*P* > 0.05) (Table 1).

Effect of EA on VIP and NO concentrations

In cold restraining stress rats, VIP and NO concentrations in plasma and mucosal and bulb tissues were obviously decreased (*P* < 0.01). Following EA of “Zusanli” (ST36), the concentrations of VIP and NO rose obviously (*P* < 0.01, *P* < 0.05) in comparison with those of the model group. In the non-acupoint group, the levels had no obvious difference compared with those of model group (Table 2).

Table 2 Effect of EA on VIP and NO concentrations under restrained-cold stress in rats (Mean \pm SD)

Groups	n	NO			VIP		
		Plasma ($\mu\text{mol}\cdot\text{L}^{-1}$)	Antrum (ng/mg)	Bulb tissues (ng/mg)	Plasma ($\mu\text{mol/L}$)	Antrum (ng/mg)	Bulb tissues (ng/mg)
Control	10	28.35 \pm 1.32	45.23 \pm 2.11	34.51 \pm 3.24	8.80 \pm 2.55	1.95 \pm 0.62	0.48 \pm 0.07
Model	10	19.24 \pm 0.95 ^b	28.01 \pm 1.42 ^b	23.48 \pm 2.62 ^b	5.16 \pm 1.58 ^b	1.12 \pm 0.46 ^b	0.37 \pm 0.05 ^b
EA	10	25.36 \pm 1.61 ^d	44.51 \pm 0.89 ^d	31.72 \pm 1.06 ^d	14.35 \pm 3.47 ^d	1.63 \pm 0.59 ^a	0.64 \pm 0.10 ^d
Non-acupoint	10	20.13 \pm 1.78	29.76 \pm 1.14	28.67 \pm 1.06	4.87 \pm 1.48	1.09 \pm 0.41	0.39 \pm 0.05

^b $P < 0.01$ vs control group; ^a $P < 0.05$, ^d $P < 0.01$ vs model group.

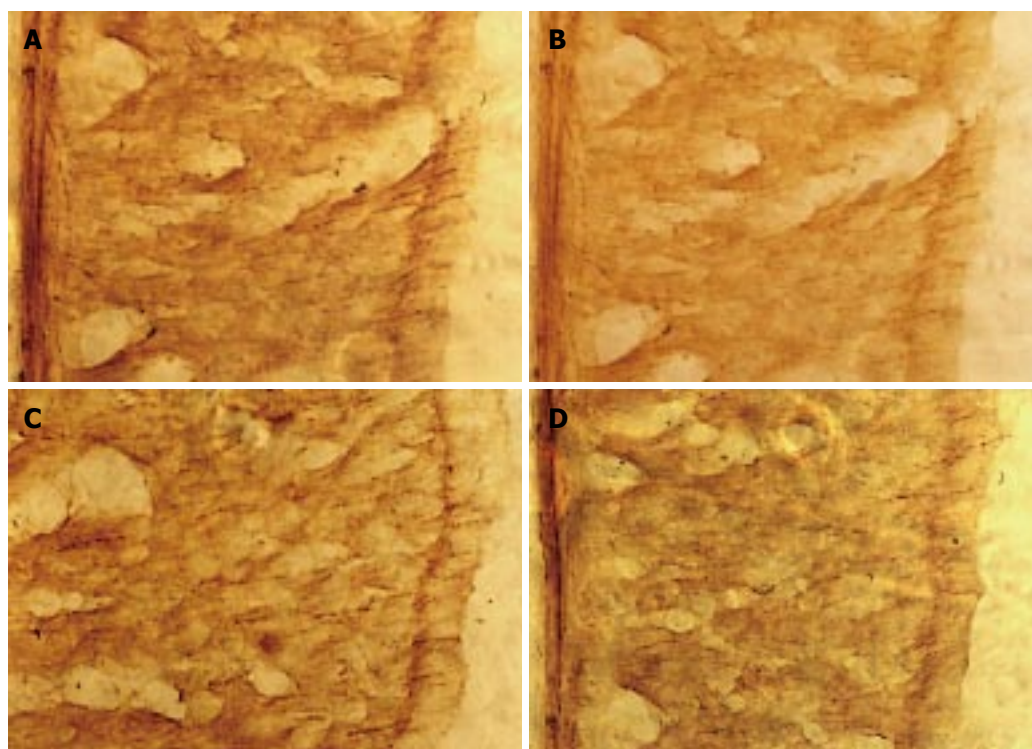


Figure 1 Microscopic photography of VIP-positive fibers of gastric smooth muscle in antrum ($\times 400$). **A:** Moderate immunoreactive staining of VIP-positive nerve fibers in control group ($\times 400$); **B:** Weak immunoreactive staining of VIP-positive nerve fibers in model group ($\times 400$); **C:** Strong immunoreactive staining of VIP-positive nerve fibers in EA group ($\times 400$); **D:** Moderate immunoreactive staining of VIP-positive nerve fibers in non-acupoint group ($\times 400$).

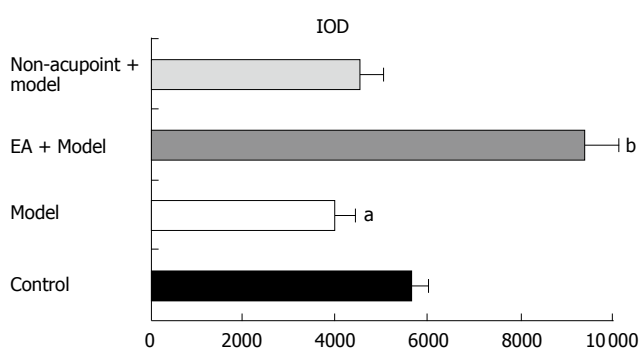


Figure 2 IOD analysis of the effect of EA on VIP immunoreactivity. ^a $P < 0.05$ vs control group; ^b $P < 0.01$ vs model group. Data are mean \pm SD, $n = 10$.

Detection of VIP by immunocytochemistry

Immunocytochemistry results showed that positive immune reaction for VIP in fibers presented as a series of beads or filaments in brown color, with a background showing no staining or stained yellow. The positive reaction was usually located in the muscle layer and submucosa. In the gastric wall muscle layer of model group, positive immune reaction for VIP fibers appeared

as lighter staining and lower density. In gastric wall muscle layer of the EA group, positive immune reaction for VIP fibers appeared as obviously dense staining (Figure 1). In the model group, computer image analysis showed that the expression of VIP in gastric wall declined obviously in comparison with the control group (3979.31 ± 582.10 vs 5646.25 ± 458.79 , $P < 0.05$), while those of VIP in gastric wall in the EA group increased obviously in comparison with model group (9420.50 ± 897.56 vs 3979.31 ± 582.10 , $P < 0.01$). However, no obvious changes of the densities of VIP in gastric wall in the non-acupoint group were found. This indicated that the effect of EA of “zusunli” can enhance the expression of VIP in gastric wall (Figure 2).

DISCUSSION

Brain gut peptides (BGPs) distribute extensively in the brain and the gastrointestinal tract. It has been demonstrated that some BGPs including gastrin (GAS), motilin (MTL), substance P(SP), VIP and somatostatin(SS) participate in gastrointestinal motility, secretion and absorption. Recent data revealed that BGPs are partially responsible for the regulatory effect of EA

on gastrointestinal tract activity, and EA can generate apparent changes in many bioactive substances such as GAS, MTL, SP, VIP, *etc.*, and the effect of EA of “Zusanli” (ST36) is of high specificity^[12,13]. Acupuncture at the stomach channel of foot-Yangming can increase the levels of NO and NOS in gastric mucosa and the NOS-positive nerves in antral myenteric plexus^[14,15]. In recent years, it has been found that decrease in gastric mucosal blood flow (GMBF) and hyperactivity of gastric motility play an important role in inducing gastric mucosal lesions under stress conditions^[16]. During stress, the gastric smooth muscle contracts intensively, resulting in disturbance of the blood circulation, decrease of GMBF and increased permeability of the vascular wall. As a result, gastric ulcer occurs^[17,18]. The results of the present study showed that in cold-restraining stressed rats, the gastric mucosal lesion was obvious. EGG was disordered and irregular. The frequency and amplitude of gastric motility were higher than that in control group ($P < 0.01$). VIP and NO levels in plasma and mucosal and bulb tissues were obviously lowered ($P < 0.01$). Following EA of “Zusanli”(ST36), the frequency and amplitude of gastric motility were lowered ($P < 0.01$), while the concentrations of VIP and NO were increased obviously ($P < 0.01$ or $P < 0.05$) in comparison with those of the model group and the expression of VIP-positive nerve fibers in gastric smooth muscle was elevated significantly ($P < 0.01$ or $P < 0.05$) in comparison with model group. The abnormal gastric motility caused by cold-restraining stress can be improved by EA and the mechanism of EA may be related to the endogenous changes of NO and VIP.

The regulation of gastric motility is a complex process related to neural activity and gastrointestinal hormone. NO and VIP, two main inhibitory neurotransmitters of the nonadrenergic noncholinergic (NANC) nerve of the intrinsic intestinal nerve system, were extensively distributed in the brain and gastrointestinal tract. They play an important role in the modulation of the function of the digestive tract. Immunocytochemistry showed that VIP-positive fibers were closely distributed around the nNOS-positive neurons. The results suggested that nNOS-positive neurons might have close morphological relationship with VIP-positive or AChE-positive neurons. They might be coordinated in the regulation of the function of the digestive tract and nitric oxide might regulate the activity of both myenteric neurons and smooth muscle^[19]. NO is involved in NANC nerve-induced relaxation and the participation of VIP (and related neuropeptides) cannot be excluded in causing relaxation of mouse gastric fundus muscle strips. Experimental findings support the idea that VIP directly stimulates the production of NO by increasing nNOS activity and thereby activating soluble guanosine cyclase in smooth muscle^[20]. It has been found that 65% of NOS containing immune active nerve fibers also have VIP immune activity, and 75% of VIP containing immune active nerve fibers also possess NOS immune activity in alimentary tract smooth muscle neurons, nerve fibers and submucosa in cats^[21]. In the rabbit stomach circular muscle layer, VIP combined with the specific receptor can lead to intracellular Ca^{2+} content increase in stomach muscle cells. Microinjection of VIP

into the dorsal vagal complex(DVC) evokes increases in gastric motor activity^[22]. Extracellular VIP also stimulates stomach muscle cells to produce NO, and increase the content of inner cAMP and cGMP, inducing muscle relaxation. Recent studies in gastric muscle strips of rabbits, rats, and guinea pigs showed that VIP-induced relaxations were inhibited by NOS inhibitors, suggesting the cascade pathway, in which VIP was proposed to be the primary neurotransmitter, inducing relaxation partially via activation of adenylyl cyclase and partially via stimulation of NO production^[23-25].

NANC nerve excitement by electric field stimulation induces muscle relaxation of gastric fundus. The underlying mechanism has been attributed to the low frequency stimulation which acts through NO production and VIP release^[26,27]. Keef *et al*^[28] proposed that NOS and VIP-like immunoreactivities are co-localized in enteric neurons and varicose fibers in the circular muscle layer, and that NO and VIP are co-transmitters, released in parallel from enteric inhibitory nerves. We conclude that NOS and VIP are extensively distributed in the nerve cells and nerve fibers of the whole alimentary tract from esophagus to anus. In the meantime, coexistence could also be seen. Zhang *et al* put forward that the mechanism of cooperative effects of VIP and NO may be as follows: NO and VIP act together as NANC nerve inhibitory transmitters. When NANC nerves excite, they induce release of VIP and increase of NO production. NO and VIP through interaction accelerate production of themselves^[29]. NO, besides producing smooth muscle relaxation, may enhance the release of VIP, which in turn may further stimulate NO formation^[30]. In contrast, the ineffectiveness of NOS inhibitors on the relaxations induced by VIP in many gastrointestinal tissues supports the idea that there is no interaction between NO and VIP in the gastrointestinal tract^[31]. Thus there is still controversy about the interaction between NO and VIP and the underlying mechanisms of this interaction remain to be resolved.

The results of this study suggest that gastric motility disorders during restrained-cold stress may be partially mediated by release of BGPs. Changes of NO and VIP levels in plasma and mucosal and bulb tissues may be related with the gastric motility. The regulative effect of EA on gastric motility is closely correlated with the increase or decrease of NO and VIP. EA at “Zusanli” acupoint (ST36) is capable of improving gastric motility function of the animal model by increasing the levels of VIP and NO. These data imply that NO and VIP may have a synergistic modulative effect on gastric mucosal blood flow and gastric motility. The modulative mechanism of acupuncture on gastroenterotract may be through the intricate neuro-endocrine-immune network.

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The timing of bowel preparation before colonoscopy determines the quality of cleansing, and is a significant factor contributing to the detection of flat lesions: A randomized study

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Abstract

AIM: To compare the cleansing quality of polyethylene glycol electrolyte solution and sodium phosphate with different schedules of administration, and to evaluate whether the timing of the administration of bowel preparation affects the detection of polyps.

METHODS: One hundred and seventy-seven consecutive outpatients scheduled for colonoscopy were randomized in one of four groups to receive polyethylene glycol electrolyte solution or oral sodium phosphate with two different timing schedules. Quality of cleansing, polyp detection, and tolerance were evaluated.

RESULTS: Patients receiving polyethylene glycol or sodium phosphate on the same day as the colonoscopy, obtained good to excellent global cleansing scores more frequently than patients who received polyethylene glycol or sodium phosphate on the day prior to the procedure ($P < 0.001$). Flat lesions, but not flat adenomas, were more frequent in patients prepared on the same day ($P = 0.02$).

CONCLUSION: The quality of colonic cleansing and the detection of flat lesions are significantly improved when the preparation is taken on the day of the colonoscopy.

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Key words: Colonoscopy; Preparation; Polyp; Flat lesion; Sodium phosphate; Polyethylene glycol

INTRODUCTION

The aim of bowel preparation before colonoscopy is to obtain a clean bowel allowing for examination of the whole mucosal surface. This preparation method should be safe and well tolerated by the patient. However, the ideal colonic lavage solution is not yet available. The two most widely employed cleansing methods are sodium phosphate (NaP) and polyethylene glycol electrolyte solution (PEG-ELS). Available data suggest that NaP achieves excellent cleansing when the first dose is administered the day before the examination and the second one a few hours before the colonoscopy^[1]. On the other hand PEG-ELS administered the same day as the colonoscopy renders a better preparation quality than when given the day before^[2]. For both preparation methods, the cecum and ascending colon seem especially susceptible to a worse preparation when the whole agent is given the day before^[1,2]. To date, no studies have been designed to compare the efficacy of NaP and PEG-ELS at their respective best administration schedules.

In the majority of available studies, the quality of bowel preparation is evaluated according to the endoscopist's impression, using pre-established scales^[1-4]. These scales only take into consideration the features and amount of remaining material. Although the detection of polyps is one of the main aims of colonoscopy, the impact of colonoscopy preparation quality on the detection of polyps has only been investigated in two recently published studies^[5,6]. Flat lesions are hard to detect due to their limited endoscopic expression. However they show a higher rate of high grade dysplasia and cancer than protruding polyps of similar sizes^[7-9], and for that reason they have become a recent field of interest and research. Although it is generally assumed by most experts in the

field that a good preparation is essential for the detection of flat lesions^[10] no prospective studies have addressed that issue. Therefore it is important to determine the influence of bowel preparation quality in the detection of flat lesions and protruding polyps, as it may have important implications in the screening of colorectal neoplasia.

The aim of the present study was to investigate whether the timing of lavage solutions before colonoscopy determines the quality of colonic cleansing and facilitates the endoscopic recognition of polyps.

MATERIALS AND METHODS

Subjects

One hundred and ninety-seven consecutive outpatients, aged between 18 and 85 years, scheduled for elective colonoscopy with morning or afternoon appointments were initially included in the study. Exclusion criteria were: pregnancy, partial or total colectomy, and inflammatory bowel disease (known or suspected). The reason for excluding patients with suspected inflammatory bowel disease was that sodium phosphate can produce mucosal changes similar to those found in Crohn's disease, which may lead to misdiagnosis. Twenty patients were not eligible for the following reasons: impossibility of reaching the cecum due to loop formation ($n = 5$), neoplastic stricture ($n = 2$), abdominal pain during colonoscopy ($n = 2$), bradycardia during colonoscopy ($n = 1$), protocol deviation due to incorrect administration of study medication ($n = 3$), incomplete data ($n = 6$), or breakdown of the endoscope during examination ($n = 1$). Therefore, one hundred and seventy-seven patients were finally included.

Methods

Having provided written informed consent, patients were assigned to one of the following four groups using a computer generated random number list. The numbers were assigned consecutively by the endoscopy assistant when the appointment for endoscopy was given. Group 1: PEG-ELS (Solución Evacuante Bohm, Laboratorios Bohm S.A, Fuenlabrada, Madrid, Spain) 3 Liters starting at 06:00 the same day of colonoscopy ($n = 43$); Group 2: NaP (Fosfosoda, Casen Fleet, Utebo, Zaragoza, Spain) 45 mL the day before (20:00) and 45 mL at 06:00 the same day of colonoscopy ($n = 45$); Group 3: PEG-ELS 3 L starting at 20:00 the day before ($n = 45$); Group 4: NaP 45 mL at 15:00 and 20:00 the day before colonoscopy ($n = 44$). Patients in groups 2 and 4 were encouraged to drink fluids liberally (at least 2 L) during the period of colonic cleansing. PEG-ELS and NaP were supplied by the manufacturers. Every patient in the study received Bysacodyl (Dulcolaxo, Boehringer Ingelheim S.A., San Cugat del Vallés, Barcelona, Spain), 15 mg. the day before colonoscopy, as it reduces the volume of PEG-ELS required for bowel preparation^[11]. A low-fibre diet (mainly avoidance of fruits and vegetables) was recommended for the day before colonoscopy to all subjects. Having completed bowel preparation, the patients were allowed to drink only clear fluids. Patients with co-morbid conditions (chronic renal failure, symptomatic ischemic heart disease, congestive heart failure, hypertension with poor pharmacological control) allocated to the groups receiving

NaP were given PEG-ELS instead (those allocated to group 2 and 4 were given preparation for groups 1 and 3 respectively), and evaluated on an intention-to-treat analysis. The study was approved by the ethical committee of the University Hospital of the Canary Islands.

Colonoscopic examinations were performed between 09:00 and 15:00 by four experienced endoscopists on staff, one of whom had received additional training in flat polyp detection. Standard colonoscopies (CF140L, Olympus Optical España S.A., Barcelona, Spain) performed the colonoscopic examinations. Indigo carmine (2-5 g/L) was applied with a spraying catheter or a syringe to the polyp at the will of each endoscopist, usually whenever a flat lesion was suspected, or to help clarify the margin or the surface features of any type of polyp. However indigo carmine was not applied in a routine way onto normal appearing mucosa to increase polyp detection yield. Flat elevated lesions were defined according to Sawada *et al*^[12] as those with a height of less than half their diameter. Flat depressed lesions were those with a central distinct depression^[9]. Protruding polyps were resected by cold or hot biopsy when ≤ 3 mm, and those larger were resected by snare polypectomy. Some sessile polyps were resected by saline-assisted polypectomy to enable complete resection. Flat elevated lesions ≤ 5 mm were resected by hot biopsy or mucosectomy, and larger lesions with mucosectomy. Flat depressed lesions amenable to endoscopic treatment were resected by mucosectomy.

The quality of bowel preparation was determined during colonoscopy by two observers (the endoscopist performing the examination, and the attending nurse) both unaware of the preparation method employed, according to the following scale: 5 excellent, (no material or liquid material covering $< 10\%$ of the mucosal surface in each location), 4 good, (liquid material or mucus covering $> 10\%$ of the mucosal surface), 3 acceptable, (small particles easy to suction), 2 fair, (solid material impossible to suction, covering $< 10\%$ of the mucosal surface), 1 poor, (solid material covering $> 10\%$ of the mucosal surface). Global quality was calculated, as the arithmetic mean of the quality in the different locations. Whenever there was a discrepancy in the evaluation, consensus was reached after discussion. The following variables were evaluated: gender, age, chronic constipation (defined as inability to pass stools at least 3 times weekly without laxatives), indication for colonoscopy, nausea, vomiting, abdominal pain, thirst, consideration of the preparation as moderately to very disgusting, procedure time (AM or PM), quality of cleansing (globally and in the different large bowel locations), polyp status (existence or absence), protruding polyp status, flat lesion status, polyp histology, and endoscopist.

Statistical analysis

In advance of the study, a statistical sample size calculation was performed based on previously published data, indicating a good to excellent preparation in 90% of patients receiving PEG-ELS or NaP on the same day of colonoscopy, and in 70% of patients receiving those preparations the day before. To achieve an absolute difference of 20% in the frequency of good to excellent quality of cleansing in the cecum and ascending colon (score ≥ 4) (with a statistical type I error of 5% and a

Table 1 Basal features of patients in the four preparation groups

	Group 1	Group 2	Group 3	Group 4	P
Patients (n)	43	45	45	44	
Gender ratio (M/F)	21:22	24:21	19:26	21:23	NS
Age (yr) (mean \pm SD)	58.0 \pm 15.9	52.4 \pm 16.7	53.6 \pm 15.2	54.0 \pm 16.5	NS
Chronic constipation (%)	17.1	26.3	24.4	27.2	NS
Polyp surveillance (%)	23.3	12.5	6.6	13.6	NS

Table 2 Comparison of rate of patients with cleansing quality ≥ 4 , between different treatment groups (mean \pm SD, %)

Group	Global	Cecum	Ascending	Transverse	Descending-sigmoid	Rectum
1	78.6 \pm 0.2	72.1 \pm 0.2	79.1 \pm 0.2	81.0 \pm 0.1	76.7 \pm 0.2	79.1 \pm 0.2
2	80.0 \pm 0.2	84.4 \pm 0.1	77.8 \pm 0.2	82.2 \pm 0.1	86.7 \pm 0.1	86.7 \pm 0.1
3	26.7 \pm 0.2 ^b	38.6 \pm 0.2 ^b	22.2 \pm 0.2 ^b	35.6 \pm 0.2 ^b	60.0 \pm 0.2 ^d	57.8 \pm 0.2 ^d
4	6.8 \pm 0.1 ^b	14.6 \pm 0.1 ^b	9.8 \pm 0.1 ^b	32.6 \pm 0.2 ^b	45.5 \pm 0.2 ^b	50.0 \pm 0.2 ^b

^b $P < 0.01$ vs Group 1, 2. (Bonferroni's correction); ^d $P < 0.01$ vs Group 2.

statistical power of 80%) at least 124 patients (62 patients per group) was calculated as required.

A global chi-square test and singly ordered Kruskal Wallis test were used to compare qualitative data. The comparison of the proportion of patients with good-excellent and those with worse preparation quality in the four groups, was initially analysed with the Chi-square test, and then group to group comparisons were done. For the adjustment of multiple contrasts, the Bonferroni correction was employed.

Continuous variables were compared with the Student's *t* test, and means and standard deviations (SD) were reported. Calculated $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Basal features of the patients are shown in Table 1; there were no differences among the four groups in terms of gender, age, chronic constipation or indication for colonoscopy. Two patients allocated to group 2 and two to group 4 received PEG-ELS due to chronic renal failure ($n = 2$), congestive heart failure ($n = 1$) and hypertension ($n = 1$), and were analyzed on an intention-to-treat fashion. There was no significant difference in the number of examinations performed by each endoscopist on patients prepared the same day or the day before. Endoscopist number 1 performed 84 (47.5%) of the 177 colonoscopies, 42 corresponding to preparation the same day and the remaining 42 to preparation the day before. Colonoscopy was performed in the morning (from 08:30 to 12:00) in 39.5%, 53.3%, 68.9%, 77.3% patients in groups 1 to 4 respectively ($P < 0.01$).

Quality of cleansing

When the four groups were compared, the quality of cleansing was different among all the segments and globally ($P < 0.001$). Per segments, in patients who received the preparation the same day (group 1, 2, Table 2) as the colonoscopy, quality was superior compared to

those prepared the day before (group 3, 4, Table 2). The global score was ≥ 4 in 78.6% \pm 0.2 %, 80.0% \pm 0.2%, 26.7% \pm 0.2%, and 6.8% \pm 0.1% patients in groups 1 to 4 respectively. Groups receiving preparation the same day had significantly better global scores ($P < 0.001$) than groups prepared the previous day. Fewer patients in group 4, compared to group 3, had global scores ≥ 4 , but the difference was not significant after the application of Bonferroni's correction. In general, in groups 3 and 4 a cleansing score ≥ 4 was more frequent in distal than in proximal colonic locations.

Poor preparation (global quality score < 2), theoretically requiring a repeat colonoscopy, was found in 12.4% (11/89) of the patients prepared the previous day but only in 2.2% (2/88) of those prepared the same day as the colonoscopy ($P = 0.02$). Poor preparation precluded insertion to cecum in two patients in group 4 and one in group 3 respectively.

Polyp detection (Tables 3 and 4)

One-hundred and eighty three polyps were detected in eighty-six (48.6%) patients. Thirty-seven (20.2%) were flat (36 flat elevated, 1 flat depressed); the total number of flat lesions detected in patients who received some preparation the same day as the colonoscopy (groups 1, 2) was significantly higher ($P = 0.002$) than in those prepared the day before (Table 3). However, no significant difference was found between groups prepared the same day or the day before in the total number of polyps, the number of protruding polyps, or the number of small (≤ 3 mm) polyps. Indigo carmine was employed in 39 (44.3%) patients in groups 1 and 2, and in 22 (24.7%) in groups 3 and 4 ($P = 0.005$). There was a histological confirmation for 152/183 (83.1%) polyps, for 79.1% and 86.6% of those detected in patients receiving preparation the day before (group 3, 4) or the same day as the colonoscopy (group 1, 2) respectively. There was one (0.6%) submucosally invasive cancer, 107 (70.4%) adenomas, and 44 (28.9%) non-neoplastic polyps. Among flat lesions, 23/31 (74.2%) were neoplastic, whereas 75/121 (70.2%) protruding polyps were neoplastic. The rate of neoplastic polyps

Table 3 Distribution by size and shape of all polyps detected in the patients who received bowel preparation the same day and the day before

Lesion	Preparation same day (group 1, 2)	Preparation day before (group 3, 4)	P
Polyp size (mean ± SD)	5.6 ± 3.8	5.3 ± 5.1	NS
Polyp size ≤ 5 mm (n = 121)	59	62	NS
Flat lesions (n = 37)	28	9	< 0.01
Flat lesions size (mean ± SD)	4.8 ± 3.7	9.0 ± 12.0	NS
Protruding polyps (n = 146)	69	77	NS
Protruding polyps size (mean ± SD)	5.9 ± 3.8	4.9 ± 3.6	NS
Total polyps (flat + protruding) (n = 183)	97	86	NS

Bonferroni's correction.

Table 4 Polyp status in patients who received bowel preparation the same day or the day before (group 3, 4), n (%)

	Preparation same day (group 1, 2)	Preparation day Before (group 3, 4)	P
Patients (n)	(88)	(89)	
Any polyp	46 (52.3)	40 (44.9)	NS
Flat lesions ¹	19 (21.6)	8 (9.0)	< 0.05
Protruding polyps ²	35 (39.7)	37 (41.6)	NS
Multiple (≥ 3) polyps	16 (18.1)	14 (15.7)	NS
Small (≤ 5 mm) polyps	39 (44.3)	33 (37.0)	NS

¹Irrespective of the existence of protruding polyps; ²Irrespective of the existence of flat polyps.

between patients prepared the same day (61/84: 72.6 %) and those prepared the day before (47/68: 69.1%) was not statistically different. A histological diagnosis was lacking in 31/183 (16.9%) of the lesions; the most frequent reasons being inadequate preparation that precluded polyp sampling, resection or recovery, or patients on whom polypectomy was not performed at that moment, but who required a repeat colonoscopy for that purpose.

Among patients with polyps, fifty-nine (68.6%) had protruding polyps, thirteen (15.2%) had flat lesions, and fourteen (16.2%) had both protruding polyps and flat lesions. Altogether in 27 (31.4%) patients who had any kind of polyp, flat lesions were detected.

For the patient-based analysis, polyp status was evaluated: polyp status positive (patients with polyps), protruding-polyp status positive (patients with protruding polyps irrespective of presence or absence of flat lesions), flat-lesion status positive (patients with flat lesions, irrespective of presence or absence of protruding polyps), multiple-polyps (≥ 3 polyps) status, and minute-polyp (≤ 3mm) status (Table 4). There was no difference in the polyp status, protruding-polyp status, or the minute or multiple polyp status; however flat-lesion status positive was more frequent in groups 1 and 2 compared to groups 3 and 4 (21.6% *vs* 9.0%, respectively, *P* = 0.02).

The following factors were significantly associated with the diagnosis of flat lesions in the univariate analysis: male

Table 5 Comparison of the tolerance variables in the four groups n (%)

Side effect	Group 1	Group 2	Group 3	Group 4	P
Nausea	17 (39.5)	24 (53.3)	24 (53.3)	15 (34.1)	NS
Vomiting	4 (9.3)	10 (22.2)	10 (22.2)	8 (18.2)	NS
Disgusting ¹	10 (23.3)	12 (26.7)	12 (26.7)	12 (27.3)	NS
Thirst ¹	6 (13.9)	9 (20.0)	9 (20)	12 (27.2)	NS
Abdominal pain ¹	4 (9.3)	4 (8.9)	3 (6.6)	5 (11.4)	NS
Anal pain ¹	1 (2.3)	4 (8.9)	1 (2.2)	2 (4.5)	NS

¹As moderate or severe.

gender, endoscopist number 1, and “same day” preparation.

Tolerance and side effects (Table 5)

There was no difference regarding the occurrence of nausea, vomiting, thirst, abdominal pain or anal pain among patients in the four groups. A similar proportion of the patients in the different groups rated the preparation as moderately to very disgusting. The proportion of patients in the different groups who had bowel activity on their way to the hospital was: 16.3%, 7.7%, 9.1% and 9.5% for groups 1, 2, 3, and 4 respectively (not statistically significant).

DISCUSSION

It has been proven that a superior quality of cleansing is obtained when PEG-ELS is administered wholly or in part on the same day as the colonoscopy^[2,13]. In fact, in many Japanese institutions patients take the PEG-ELS in the endoscopy waiting room and the examination is started when the excretions become liquid and transparent. Similarly, significantly better cleansing scores have been reported when the second dose of NaP is given on the same day as the colonoscopy^[11,3]. Although many studies have compared the efficacy of cleansing between PEG-ELS and NaP, PEG-ELS was given in those studies on the day before the colonoscopy. To the best of our knowledge this is the first study ever to compare oral PEG-ELS and NaP administered at their respective best timing schedules, and our results confirm the importance of timing. In the current study, when the agent was administered the same day as the colonoscopy (the whole agent for PEG-ELS or partial for NaP), the quality of cleansing was significantly better globally and per segment than when the whole preparation was given the day before. In addition, the detection of flat lesions was markedly improved in patients with a better quality of cleansing, given that the number of flat lesions were significantly greater in patients receiving the bowel cleansing agents the same day than in those prepared the day before.

In our study the groups receiving preparation the same day as the colonoscopy reached a good to excellent quality of cleansing for all colonic segments. It is important to note that more than 75% of patients prepared the same day had a global score of ≥ 4 (good-excellent). However in groups prepared the day before, proximal bowel segments were especially poorly prepared. This

would indicate that having obtained a clean colon, material coming later from the small bowel could make the colon become dirty again, starting with the right colon. There are at least two possible explanations for that fact. Firstly, gastric, intestinal and biliary secretions could account for the material staining the colon. Secondly, although bowel preparation methods clean the colon efficiently, their effect on the small bowel might be more modest. In both instances, a longer time interval between the administration of the bowel preparation agent and the colonoscopy could account for a dirty colon. Videocapsule or enteroscopy studies could be useful to determine small and large bowel cleansing quality with different timing of administration of preparation agents.

In Spain both PEG-ELS and NaP are employed for colonoscopy. NaP is usually administered in the same way as in our study; however PEG-ELS is given by most institutions the day before colonoscopy, probably because of concern regarding the duration of diarrhoea after its ingestion. In the current study colonoscopies were scheduled from 09:00 to 14:00, and this is the rule for most public hospitals in our country. Although significant differences were not found among the different groups, we found that 16.3% of those in group 1 (PEG-ELS the same day) had bowel movements on their way to the hospital. Therefore, it might be reasonable to administer PEG-ELS at least one hour earlier than we did in our study. Although such an early administration will interfere with the patient's sleep, we believe that the effort is worthwhile, providing a good quality of cleansing and diagnostic yield, and preventing repeat examinations. Although it could be argued that from the patient's perspective it might be more difficult to ingest a large volume of fluids a few hours before the examination (same day), than the day before, no difference in tolerance variables was found between the groups. Conversely, it could be said that drinking the bowel prep a few hours before the examination, instead of the previous day, would impact on the patient's activities during a shorter period of time. Moreover, in the present study bisacodyl was used in order to reduce the total volume of PEG-ELS, as it has proven to be effective for that purpose^[11]. In our opinion, the remarkably significantly superior preparation quality observed in patients prepared the same day seems to justify that choice. An alternative strategy could be to give the bowel preparation in the endoscopy unit, which is a common practice in many Japanese institutions.

One potential limitation of the present study is the use of a non-validated scale for assessing the adequacy of the bowel preparation. There is no standardized system for describing bowel preparation^[14], and in fact most studies comparing different methods of bowel cleansing have used non-validated scales. We used a scale similar to others employed in previous reports^[4,15], but including a numerical value (90%) to describe the average of the mucosal surface covered by liquid or faecal content. Moreover, quality assessment was done by two examiners. Therefore, although employing a validated scale would have provided more reliable data, we do not believe that this has affected our study significantly.

Colorectal cancer is the second leading cause of cancer

death in most western countries, and the detection and endoscopic removal of colonic polyps has been proven to reduce the incidence of colorectal cancer. The polyp 'miss' rate seems to be especially high for lesions < 10 mm, as determined by tandem colonoscopy studies^[16]. Flat lesions represent about one fourth of all colonic polyps and may be hard to detect at colonoscopy. In the present study, flat lesions were more frequently detected in patients receiving PEG-ELS or oral NaP the same day as the colonoscopy, who reached higher rates of good to excellent bowel cleansing. The impact of colonic cleansing on polyp detection had been assessed only in two previous studies. Harewood *et al*^[5], in a retrospective study including more than 90000 colonoscopic procedures, found that an adequate preparation was associated with the detection of small (≤ 9 mm) polyps, but not of larger polyps. In a prospective multicenter European study, the detection of polyps of any size, but not of cancer, was dependent on cleansing quality^[6]. The present study represents the first evidence that flat lesion detection is associated with quality of cleansing. Although flat lesions were more frequent in patients prepared the same day, no significant difference was detected when flat neoplasia was evaluated; in fact the lack of histological diagnosis in 17% of the polyps is a potential limitation of our study. However, 74% of flat lesions with histological diagnosis available were neoplastic, which is similar to previously reported^[7]. Although an increased detection of small flat hyperplastic lesions can be expected with a high quality of cleansing, it is well known that minute flat adenomas can bear high grade dysplasia and even invasive cancer; an excellent cleansing seems therefore desirable to improve the detection of such lesions.

The more frequent application of indigo carmine in the groups receiving preparation the same day might be interpreted as another limitation of our study, as it has been proven that pancolonial application of indigo carmine with a spraying catheter increases the detection rate of small polyps^[17,18]. However, as explained in the materials and methods section, indigo carmine was applied only when a flat lesion was suspected, and only in a small amount on the suspect area. On the other hand, the more frequent application of indigo carmine in well-prepared patients (those in groups 1 and 2) is not surprising, as chromoendoscopy is used only when the colonic mucosa is sufficiently clean^[19]. Therefore, we believe that the effect of indigo carmine did not play a significant role in the increased detection of flat lesions in groups 1 and 2 in our study.

Nevertheless the outcome of real interest would be the detection of flat adenomas, rather than of flat lesions in general. The impact of the timing of bowel preparation and colonic cleansing quality on flat neoplasia detection should be evaluated in future large-scale randomized studies, adequately powered for that purpose. The importance of other factors presumably involved in flat lesion detection, such as the expertise of the endoscopist and use of chromoendoscopy should be ascertained.

Apart from an excellent bowel preparation, flat lesion detection requires a high suspicion index by the endoscopist including specific training in their detection^[20].

In fact this contention was also proven in our study. Although the four participating endoscopists shared a similar number of explored patients in each study group, endoscopist 1, who had additional training in chromoendoscopy and flat lesion detection, identified significantly more flat lesions than the others. The basic techniques for flat lesion detection, interpretation and treatment should constitute a part of the training in colonoscopy. In the present study, both preparation methods were well tolerated, and we found no difference in this respect, although other studies indicated that NaP is tolerated better due to its smaller volume^[1,3,4,15,21-24]. However in recent studies the amount of oral liquids with NaP has been increased (up to 3.8 liters) in order to prevent hydroelectrolytic disturbances^[25].

In conclusion, the present study demonstrates that the quality of colonic cleansing is significantly improved when the preparation (either PEG-ELS or NaP) is given a few hours before the colonoscopy. Although there was no overall difference in the number of polyps detected, flat lesions were more frequent in patients receiving preparation the same day. Training of the endoscopist in flat lesion detection seems to influence their detection. Larger prospective studies are needed to determine the impact of colonic preparation quality on the detection of flat and protruding neoplasia.

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Abnormalities of uterine cervix in women with inflammatory bowel disease

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Abstract

AIM: To evaluate the prevalence of abnormalities of the uterine cervix in women with inflammatory bowel disease (IBD) when compared to healthy controls.

METHODS: One hundred and sixteen patients with IBD [64 with Crohn's disease (CD) and 52 with ulcerative colitis (UC)] were matched to 116 healthy controls by age (+/- 2 years) at the time of most recent papanicolaou (Pap) smear. Data collected consisted of age, race, marital status, number of pregnancies, abortions/miscarriages, duration and severity of IBD, Pap smear results within five years of enrollment, and treatment with immunosuppressive drugs. Pap smear results were categorized as normal or abnormal including atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesion (LGSIL), and high-grade squamous intraepithelial lesion (HGSIL).

RESULTS: The median age at the time of Pap smear was 46 (range: 17-74) years for the IBD group and matched controls (range: 19-72 years). There were more Caucasian subjects than other ethnicities in the IBD patient group ($P = 0.025$), as well as fewer abortions ($P = 0.008$), but there was no significant difference regarding marital status. Eighteen percent of IBD patients had abnormal Pap smears compared to 5% of controls ($P = 0.004$). Subgroup analysis of the IBD patients revealed no significant differences between CD and UC patients in age, ethnicity, marital status, number of abortions, disease severity, family history of IBD, or disease duration. No significant difference was observed

in the number of abnormal Pap smears or the use of immunosuppressive medications between CD and UC patients ($P = 0.793$). No definitive observation could be made regarding HPV status, as this was not routinely investigated during the timeframe of our study.

CONCLUSION: Diagnosis of IBD in women is related to an increased risk of abnormal Pap smear, while type of IBD and exposure to immunosuppressive medications are not. This has significant implications for women with IBD in that Pap smear screening protocols should be conscientiously followed, with appropriate investigation of abnormal results.

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Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Cervical cancer; Pap smear

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INTRODUCTION

Cervical cancer is responsible for almost 4000 deaths annually in the United States^[1]. Due in large part to mass screening protocols with papanicolaou (Pap) smears, mortality from cervical cancer has declined by over 70% in the past 50 years^[2]. The importance of mass cervical cytology screening programs in early detection of pre-cancerous and cancerous lesions has been well established. An increase in Pap smear abnormalities has not been documented in patients with either ulcerative colitis (UC) or Crohn's disease (CD), although anecdotal reports have suggested such an association. A previous study of hospitalized patients at Lenox Hill Hospital has reported a higher rate of Pap smear abnormalities in inflammatory bowel disease (IBD) patients (47%) compared to controls (15%)^[3].

The purpose of the present study was to compare the rate of cervical abnormalities in non-hospitalized patients with IBD *versus* age-matched controls and to evaluate whether

type of IBD (UC *vs* CD) and use of immunosuppressive medications are related to cervical pathology.

MATERIALS AND METHODS

This was a retrospective cohort study approved by the institutional review board. The study was performed at Lenox Hill Hospital as an interdepartmental collaborative effort amongst gastroenterologists, obstetrician-gynecologists and pathologists. Patients with IBD were identified from an IBD database that spans 30 years, which was established by the Director of Gastroenterology (BIK). Patients were contacted by mail, phone or during office visits and asked to complete questionnaires, which required information regarding age, ethnicity, marital status, type and duration of IBD, severity of illness, number of pregnancies/abortions, family history of IBD, and IBD medications [specifically the use of the immunosuppressive drugs 6-mercaptopurine (6-MP) and azathioprine (AZA), and the anti-TNF drug infliximab] used at time of PAP smear. They were 18-80 years of age with confirmed IBD and required at least one Pap smear in the previous five years for inclusion in the study. Exclusion criteria were other immunosuppressed states, such as human immunodeficiency virus (HIV), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) positivity. Patients signed IRB-approved release forms in order to collect the Pap smear results from their respective gynecologists. If the IBD cases received treatment with 6-MP, AZA, and/or infliximab, the duration of therapy with these drugs was calculated until the time of the Pap smear result available within the time-frame of our study (retrospective 5 years). Data regarding the use of steroids and 5-ASA products were collected using our internal IBD database, supplemented by personal communication. Severity of IBD was determined by subjective patient opinion of the disease, rated as mild, moderate or severe.

Age-matched controls (± 2 years) were identified utilizing demographic information from participating gynecologists. Controls were asked to complete questionnaires at the time of routine gynecological office visits. Inclusion and exclusion criteria were identical to the cases, except for presence of IBD. Pap smear results were obtained at the time of inclusion for the controls. For the IBD group, we contacted the patients' gynecologists in person or via telephone and requested Pap smear results and, if available, human papillomavirus (HPV) status for five years prior to the study. For the cases diagnosed with IBD within the past 5 years, we included the Pap smear results that were obtained subsequent to the diagnosis. Pap smear results were considered abnormal with any of the following: atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesion (LGSIL), high-grade squamous intraepithelial lesion (HGSIL), which includes carcinoma *in-situ*. When the Pap smear was abnormal, we requested any subsequent pathology reports from gynecological procedures, including loop electrosurgical excision procedure (LEEP), cone biopsy, and/or hysterectomy.

Table 1 Patient demographics: IBD *vs* controls

Patient demographics	Total IBD <i>n</i> = 116	Controls <i>n</i> = 116	<i>P</i>
Median age (yr) (range)	46.5 (17-74)	46 (19-72)	
Ethnicity			0.025
Caucasian	93%	85%	
Other (AA, Asian, Hispanic, Indian, Multicultural, West Indian)	7%	15%	
Marital status			0.4
Single	21%	18%	
Married	70%	64%	
Divorced	7%	12%	
Other (widow, unknown)	3%	6%	
Abortions (<i>n</i>)			0.008
0	80%	65%	
1	11%	17%	
> 1	9%	18%	
Disease severity ¹			
Mild	56%		
Moderate	31%		
Severe	12%		
Nonresponders	2%		
Family history of IBD ²			
Yes	37%		
No	61%		
Unknown	3%		
Disease duration in years			
Median (yr) (range)	16 (1-55)		
6MP use			
<i>n</i>	55		
Median (yr) (range)	6 (1-26)		
Imuran use			
<i>n</i>	9		
Median (yr) (range)	3 (1-7)		
Infliximab use			
<i>n</i>	12		
Median (yr) (range)	1 (1-2)		

¹Combined patient-physician assessment; ²Includes at least one other first degree or second degree family member with IBD.

Statistical analysis

Demographic and clinical characteristics were obtained for both cases and controls. The statistical analysis plan included the calculation of descriptive statistics, such as percentage and median (range) for categorical and continuous data. Comparisons on non-parametric (categorical) data between the groups were made with the McNemar test for matched samples (IBD *vs* controls) and with the Pearson chi square test with Yates correction for independent samples (UC *vs* CD). $P < 0.05$ was considered statistically significant. SPSS (version 14.0) statistical software was utilized for all analyses.

RESULTS

A total of 116 cases were enrolled in the study. Of these, 91 were recruited *via* mail and 30 were recruited in person. Five cases were excluded utilizing the exclusion criteria. All the 116 age-matched controls were recruited in person. The demographic information is shown in Table 1 (IBD *vs* healthy controls) and Table 2 (subgroup analysis of IBD, CD *vs* UC). The groups were statistically different in

Table 2 Patient demographics: CD *vs* UC

Patient Demographics	CD 64	UC 52	P
Median age (yr) (range)	47 (20-72)	45 (17-74)	0.94
Ethnicity			0.666
Caucasian	59	49	
Other (AA, Asian, Hispanic, Indian, Multicultural, West Indian)	5	3	
Marital status			0.914
Single	14	10	
Married	44	38	
Divorced	4	3	
Other (widow, unknown)			
Abortions (n)			0.901
0	51	42	
1	8	5	
> 1	5	4	
Disease severity ¹			0.2
Mild	31	33	
Moderate	25	13	
Severe	8	6	
Family history of IBD ²			0.14
Primary	18	10	
Secondary	10	4	
No family history	34	37	
Disease duration in years			
Median (yr) (range)	19 (1-55)	14 (1-40)	0.147
6MP use			
n	39	16	
Median (yr) (range)	6 (1-26)	6 (1-12)	0.628
Imuran use			
n	6	3	
Median (yr) (range)	4.5 (2-7)	3 (1-4)	0.433
Infliximab use			
n	10	2	
Median (yr) (range)	1 (1-2)	1 (1)	0.392

¹Combined patient-physician assessment; ²Includes at least one other first degree or second degree family member with IBD.

ethnicity, with more Caucasian subjects in the IBD group ($P = 0.025$). There were no significant differences between the two groups with respect to marital status ($P = 0.4$). The control group had a significantly higher number of abortions ($P = 0.008$). More than half of patients within the IBD subgroups had self-reported mild disease (33/52 with UC and 31/64 with CD). One third of the patients with IBD had family clustering of the disease, with at least one other first- or second-degree relative diagnosed with IBD. There were no significant differences between the UC and CD groups with respect to age ($P = 0.94$), ethnicity ($P = 0.66$), marital status ($P = 0.90$), number of abortions ($P = 0.20$), disease severity ($P = 0.14$), family history of IBD ($P = 0.14$), duration of disease ($P = 0.147$) and length of drug therapy, including 6-MP ($P = 0.62$), AZA ($P = 0.43$), infliximab ($P = 0.39$), corticosteroids and 5-aminosalicylate (5-ASA) products.

The main results of the study are presented in Tables 3 and 4. Table 3 shows IBD *versus* controls. Within the IBD group, 18% (21/116) of Pap smears were abnormal compared with 5% (6/116) in healthy controls ($P = 0.004$). The Pap smear abnormalities found in IBD patients were ASCUS: 9.5% (11/116), LGSIL: 5.2% (6/116) and HGSIL: 3.4% (4/116). The control group had 1.7%

Table 3 PAP results in IBD patients *vs* controls

Pap results	IBD total	Controls	p
Normal	95	110	
Abnormal	21	6	
ASCUS ¹	11	2	
LGSIL ²	6	3	
HGSIL ³	4	1	
Total	116	116	
Abnormals (%)	18.1	5.2	0.004

¹Atypical Squamous Cells of Undetermined Significance; ²Low-grade squamous intraepithelial lesions; ³High-grade squamous intraepithelial lesions (including carcinoma *in situ*).

Table 4 PAP results in IBD subgroups: CD *vs* UC

Pap results	CD	UC	p
Normal	50	45	
Abnormal	14	7	
ASCUS ¹	7	4	
LGSIL ²	4	1	
HGSIL ³	3	2	
Total	64	52	
Abnormals (%)	22	13	0.793

¹Atypical Squamous Cells of Undetermined Significance; ²Low-grade squamous intraepithelial lesions; ³High-grade squamous intraepithelial lesions (including carcinoma *in situ*).

Table 5 HPV Data in IBD patients *vs* controls (for abnormal Pap results)¹

HPV status	IBD total	CD	UC	Controls
Positive (high risk)	5	5	0	2
Positive (low risk)	3	0	3	0
Negative	4	4	0	2
Unknown	9	5	4	2
Total	21	14	7	6

¹HPV data were available in a small subset of abnormal Pap results and in none of the normal Paps.

(2/116), 2.6% (3/116) and 0.9% (1/116) Pap smear abnormalities, respectively. The subgroup analysis of IBD patients shown in Table 4 indicated that 14/50 patients with CD (22%) and 7/45 patients with UC (13%) had abnormal Pap smears. This was not a significant difference ($P = 0.793$).

Table 5 represents the HPV status of abnormal Pap smear results for patients in the IBD and control groups. HPV was positive in 11 of the IBD patients (8 CD and 3 UC) and 2 of the controls, while 4 IBD patients with CD were negative for HPV. There were 9 IBD patients (5 CD and 4 UC) with unknown HPV status. The scarcity of HPV data (more than 50% of abnormal Pap results in both IBD and control groups available and none for the normal Pap outcomes) represents the changing standards of gynecological practice during the last 6 years, the time frame used in this study. Although HPV status could have been extrapolated from the pathology slides, our study

did not include consent for the destruction of Pap smear slides. As a result, no further statistical analysis could be applied and no definitive conclusion could be reached regarding presence of HPV and abnormal Pap results in this study.

Table 6 presents the association between the major medications prescribed for patients with CD and UC and Pap smear outcomes. There was no significant association between the groups with abnormal Pap smears who were on these medications and the groups with abnormal Pap smears who were not on these medications ($P > 0.38$).

DISCUSSION

The obstetrical-gynecological issues related to fertility, pregnancy, influence of drug therapy and fistulae are known entities in patients with IBD^[4], but little is known about the link between IBD and uterine cervical pathology. Other immunosuppressed disease states such as HIV^[5,6], SLE^[7,8], and organ transplantation^[9-11] have been shown to carry increased risk for developing abnormalities of the uterine cervix leading to cervical carcinoma and it is crucial to follow the recommended screening guidelines in these patient populations. In a previous study of hospitalized IBD patients, a much higher rate of cervical abnormalities is found in patients with IBD (47%) compared with non-IBD controls (15%)^[3], for which the severity of IBD symptoms leading to the hospitalization may be responsible. Furthermore, little has been reported regarding immunosuppressive medications and the risk of cervical abnormalities, but a recent study showed that infliximab use does not increase the risk of abnormal Pap smears^[12].

The results obtained through this study of 232 women demonstrate that the presence of inflammatory bowel disease is correlated with abnormal cervical histology. The group with IBD (116) and the healthy control group (116) were well matched demographically and by gynecological history, except for the higher number of Caucasians and the lower number of abortions in the IBD group. This significantly different gynecological history would have theoretically led to an outcome such that the IBD group with a lower rate of abortions should have shown a lower rate of abnormalities on Pap smear. However, the results of our study indicate that despite this significant difference in abortion rate, the IBD group still shows a significantly higher rate of Pap smear abnormalities.

Within the IBD group, the patients with CD and UC were well matched by duration and severity of disease and medication use. Our data indicate that IBD, a group of autoimmune diseases with or without immunosuppression, also carries a higher risk for developing cervical abnormalities, as more than 18% of patients in IBD group had abnormal pap smears compared to 5% in the matched control group ($P = 0.004$) over the 5-year time period. Subgroup analysis between CD and UC revealed no statistically significant difference ($P = 0.793$) in the rate of cervical pathology. Furthermore, there were no significant associations between Pap smear abnormalities and treatments with commonly used medications for IBD patients. Specifically, oral or intravenous steroids

Table 6 Incidence of abnormal pap results in 116 IBD patients after treatment

Pap results	Normal pap n (%)	Abnormal pap n (%)	Total (n)	P
Corticosteroids	49 (80)	12 (20)	61	0.747
No corticosteroids	43 (83)	9 (17)	52	
5-ASA ¹	88 (82)	20 (19)	108	0.934
No 5-ASA ¹	4 (80)	1 (20)	5	
Immunosuppressives ² and Anti-TNF α^3	54 (79)	14 (21)	68	0.381
No immunosuppressives ² and no Anti-TNF α^3	42 (86)	7 (14)	49	

¹5-aminosalicylates (sulfasalazine, mesalamine, olsalazine, etc); ²Monoclonal antibody to Tumor Necrosis Factor α (infliximab); ³6-mp and/or azathioprine.

($P = 0.747$), 5-ASA products ($P = 0.934$), and various combinations of infliximab, azathioprine, and 6-MP ($P = 0.384$) did not significantly contribute to cervical abnormalities in IBD patients. These data provide preliminary support to the conclusion that IBD may be a significant variable for developing an abnormal Pap smear.

Due to the nature of a retrospective design we have identified limitations of our study. We relied on previous Pap smear results obtained from multiple institutions, leading to interobserver variability in the interpretation of the smears. Furthermore, clinical information was obtained from patients' questionnaires, which made the assessment of disease severity and medication use difficult due to recall bias. By relating IBD severity to the timing of Pap smears, we might have been able to determine the causal effect of diarrhea on Pap smear results, but again due to the retrospective nature, this was not possible. In addition, during the time frame of our study, only a small percentage of cases and controls had HPV data available, precluding the ability to make a correlation with this contributing factor.

Despite the limitations of the study design, our data show a significant correlation between IBD and cervical abnormalities, which is not influenced by the use of immunosuppressive medications. The precise mechanism for this correlation is unknown. Other autoimmune diseases, such as rheumatoid arthritis, may also have this correlation and further research into uterine cervical abnormalities with other immunosuppressed disease states is needed. Based on our results, we recommend the standard cervical cancer screening for all IBD female patients with strict follow-up of those with abnormal findings.

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RAPID COMMUNICATION

Loss of interstitial cells of Cajal network in severe idiopathic gastroparesis

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INTRODUCTION

Gastroparesis syndrome is a clinical entity characterized by chronic nausea, epigastric discomfort and recurrent vomiting, in the absence of mechanical obstruction^[1]. Gastroparesis may be either primary (idiopathic) or secondary, i.e. associated with illnesses or specific disorders that are the likely cause^[2]. Apart from common secondary causes of gastroparesis, such as diabetes mellitus and gastric surgery, most cases are idiopathic. Although in recent years some previously-labeled "idiopathic" gastroparesis cases have been shown to have a probable causal relationship with prior viral infections^[3] or myoenteric plexus ganglionitis^[4], in most cases a definite cause or even a possible link remains undetected.

Indirect evidence from electrogastrographic^[5-7] and manometric^[8,9] studies suggests that, at least in some patients, gastric hypomotility may be due to alteration of mechanisms controlling the motor activity of the stomach. Interstitial cells of Cajal (ICC), whose importance has been suspected for decades due to their close anatomical relationship with smooth muscle cells and terminal varicosities of enteric nerves^[10-12], are the pacemaker cells in the gastrointestinal tract^[13-16] and can mediate input from the enteric nervous system^[17,18]. It may thus be expected that disruption of ICC networks leads to disordered gastrointestinal motility in the affected segments^[19]. Research into ICC biology has been greatly stimulated by the discovery that they express the proto-oncogene *c-kit*^[20,21], and activation of Kit tyrosine kinase receptor signaling by the natural ligand stem cell factor (SCF or steel)^[22,23] is necessary for development and maintenance of ICC phenotype^[15,24].

Since ICC can express Kit, immunohistochemical analysis using anti-Kit antibodies provides an efficient means of identifying them in a variety of species, including humans^[25-27]. In the last few years several reports have demonstrated that ICC abnormalities are associated with some human upper-gut pathological conditions^[28,29], although few studies in gastroparetic patients to date are available.

An abnormal reduction of pacemaker cells in the stomach has been recently described in a murine model of

Abstract

AIM: To report a case of severe idiopathic gastroparesis in complete absence of Kit-positive gastric interstitial cells of Cajal (ICC).

METHODS: Gastric tissue from a patient with severe idiopathic gastroparesis unresponsive to medical treatment and requiring surgery was analyzed by conventional histology and immunohistochemistry.

RESULTS: Gastric pacemaker cells expressing Kit receptor had completely disappeared while the local level of stem cell factor, the essential ligand for its development and maintenance, was increased. No signs of cell death were observed in the pacemaker region.

CONCLUSION: These results are consistent with the hypothesis that a lack of Kit expression may lead to impaired functioning of ICC. Total gastrectomy proves to be curative.

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Key words: C-kit; Gastroparesis; Interstitial cells of Cajal; Stem cell factor

Battaglia E, Bassotti G, Bellone G, Dughera L, Serra AM, Chiusa L, Repici A, Mioli P, Emanuelli G. Loss of interstitial cells of Cajal network in severe idiopathic gastroparesis. *World J Gastroenterol* 2006; 12(38): 6172-6177

gastroparesis^[30] and in a case of idiopathic gastroparesis^[31]. Here we report a case of severe idiopathic gastroparesis with complete absence of Kit-positive gastric ICC.

MATERIALS AND METHODS

Case history

A 39-year old woman was investigated for a long-lasting complaint of severe dyspeptic symptoms. Her past history included repeated upper GI series and endoscopic examinations, always unremarkable in 1989-1995. She was treated with antacids, sucralfate, clebopride and ranitidine, with little or no success. In 1996 she received our attention for persisting dyspeptic symptoms associated with gastro-esophageal reflux. Physical examination and blood chemistry were unremarkable. An upper panendoscopy showed antral erythema and erosions, and histologic specimens revealed the presence of active gastritis associated with *H pylori* colonization. After eradication therapy, the patient continued to complain of severe post-prandial discomfort and epigastric fullness, sometimes associated with food regurgitation. Esophageal manometry and pH-metry were normal. Scintigraphic gastric emptying of a 300 kcal standard meal (Tc-albumin labeled egg white)^[32] was severely delayed (the $t_{1/2}$ at 120 min was 474, normal values 60-150). Abdominal ultrasound scans, small bowel enema and nuclear magnetic resonance of the brain and brainstem were normal. No autonomic dysfunction was documented. Therapeutic courses of high dose cisapride and domperidone were ineffective, as were oral and intravenous erythromycin.

In 1997, since the symptoms continued to worsen with the appearance of vomiting and weight loss, a percutaneous jejunostomy was thus required for feeding. However, in March 2000, due to a poor quality of life, she was again admitted and an upper gastrointestinal manometry^[33] showed almost no fasting and post-prandial gastric motility, whereas duodenojejunal motor activity was within normal limits. A total gastrectomy with esophago-jejunal anastomosis and Roux-en-Y reconstruction was carried out. The post-surgical period was uneventful and she was discharged after 10 d. She subsequently enjoyed a good health, regained body weight, and did not complain of abdominal problems at follow-up.

Histology and immunohistochemistry

Multiple full-thickness samples from the stomach (fundus, corpus, and antrum) were obtained at surgery, and processed for conventional histological examination (H&E, Gomori's trichrome) and immunohistochemistry. The myenteric plexuses were assessed with a rabbit polyclonal anti-S-100 antibody (Dako, Carpinteria, CA, USA, dilution 1:4000), a monoclonal anti-CD56 antibody (clone 1B6, Novocastra Laboratories, Newcastle-upon-Tyne, UK, dilution 1:50), a rabbit polyclonal anti-neuron specific enolase (NSE) antibody (Dako, dilution 1:1000), and a monoclonal anti-neurofilament antibody (clone 2F11, Immunon, Shandon, Pittsburgh, PA, prediluted). Expression of Kit and SCF was assessed using a rabbit polyclonal antibody (Dako, dilution 1:50) and a goat polyclonal antiserum produced against a peptide mapping

at the amino terminus of SCF of human origin (Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1:100), respectively. Briefly, consecutive formalin-fixed and paraffin-embedded sections were dewaxed and rehydrated through alcohol series up to distilled water. Sections were subjected to heat-induced epitope retrieval (CD56, neurofilaments, Kit and SCF) by immersion in a heat-resistant container filled with citrate buffer solution (pH 6.0) placed in a pressure cooker and micro-waved for 20 min. Endogenous peroxidase activity was suppressed by incubation with 3% solution of H₂O₂ for 5 min. Immunostaining for CD56, Kit and SCF was done with a peroxidase-based visualization DAKO EnVision™ kit, following the manufacturer's recommendations. Immunostaining for S100, NSE, and neurofilaments was performed with a peroxidase-based visualization DAKO LSAB® kit, following the manufacturer's recommendations. Diaminobenzidine tetrahydrochloride was used as chromogen. The slides were then counterstained with Mayer hematoxylin for 5 s, dehydrated and mounted in Clarion (Biomed, Foster City, CA, USA).

To determine the non-specific staining, peptides blocking polyclonal antibody binding were used or sections were incubated in the absence of primary antibody. In these cases, no immunostaining was detected. Kit-positive mast cells serving as an internal control, were counted under a light microscope at × 200 magnification within a square micrometer and expressed as mean (SE) per surface area (1 mm²). Quantification of Kit-positive ICC and nervous structures was performed by manual procedures under optical microscope (Carl Zeiss-Axioscop, Germany) and computer-assisted image analysis system (Immagini&Computer, Bareggio, Milano, Italy) using the Image Pro Plus program (Media, Cybernetics, Silver Spring, MD, USA). Five areas (1 mm²) were selected from similar regions in patient and control tissues. The area of immunopositive cells was calculated and expressed as a mean percentage of the total area of the image.

SCF staining patterns were evaluated using the immunoreactive score (IRS) proposed by Remmele^[34] in which IRS = staining intensity (SI) × percentage of positive cells (PP). SI was determined as 0 = negative, 1 = weak, 2 = moderate, 3 = strong. PP was defined as 0 = negative, 1 = 1%-20% positive cells, 2 = 21%-50% positive cells, 3 = 51%-100% positive cells. Ten visual fields from different areas of each specimen were chosen at random for IRS evaluation, and the average (SE) was calculated.

Apoptosis detection

Apoptosis was detected by transferase-mediated digoxigenin-tagged 16-desoxy-uridine-triphosphate nick-end labeling (TUNEL) assay, using the DeathEnd™ colorimetric TUNEL system (Promega, Madison, WI, USA). After deparaffinization and rehydration, sections were digested with 20 mg/L proteinase K (Sigma, St Louis, MO, USA) for 15 min at room temperature, and then incubated with a terminal transferase plus nucleotide mixture for 1.5 h at 37°C. After exposure to antidigoxigenin peroxidase for 45 min, diaminobenzidine (Sigma) was used as chromogen to detect TUNEL-positive cells, and the sections were counterstained with hematoxylin.

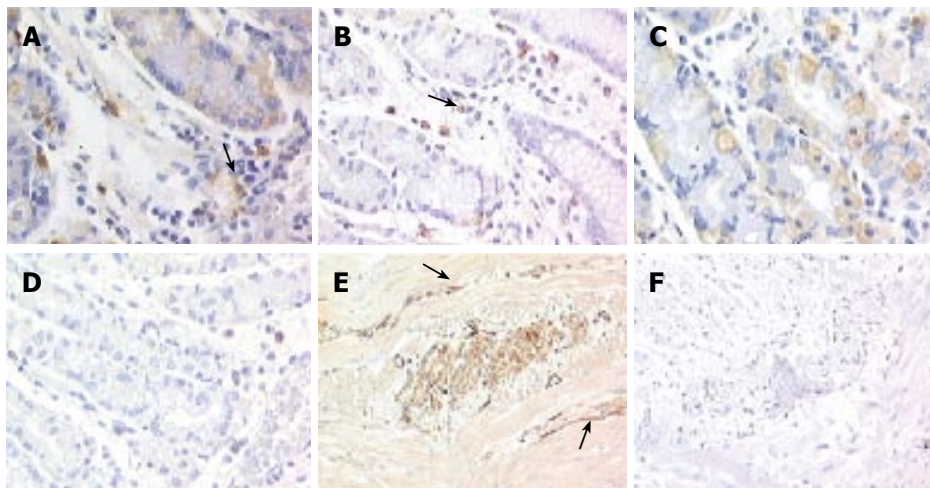


Figure 1 Expression of Kit in normal gastric tissue and gastroparesis (x 200). In both normal (A) and gastroparetic stomach (B), Kit was detected on mast cells (arrows) used as an internal control; In normal gastric samples, positivity is shown on parietal cells (C) and at the intrinsic innervation level (E, arrows), whereas in gastroparesis no staining is present at either levels (D, F).

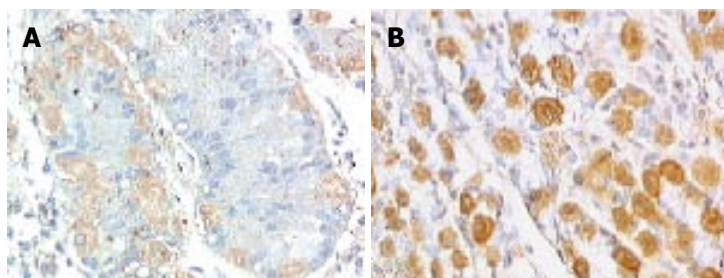


Figure 2 Expression of SCF in normal gastric tissue (A) and gastroparesis (B) (x 200). Intense intracytoplasmic expression of SCF with membrane reinforcement in gastroparesis parietal cells was present.

Controls

Control gastric tissue was obtained from 4 specimens of normal stomach taken during pancreatectomy for tumors of the pancreatic head.

Determination of SCF concentrations in sera

Serum samples collected from the patient prior to surgery and 10 age-matched healthy female volunteers, were assayed for soluble SCF using a commercially-available ELISA kit (R&D Systems, Abingdon, UK), following the manufacturer's instructions. All samples were evaluated in duplicate. The lower threshold of detection of this assay is 9 ng/L.

RESULTS

Histological examination

The mucosal, submucosal, and smooth muscle architecture appeared normal at conventional H&E staining. Trichrome stain revealed mild and scattered fibrosis of the submucosa plus some fibrosis of the external longitudinal mucosal layer as well. Neither intranuclear or viral inclusions nor any acute inflammatory cells were observed.

Immunohistochemistry

The submucous and myenteric plexus cells were abnormal in number and distribution, with ganglion cells reduced in number and size compared to normal counterparts (1.4% *vs* 3.9%). Kit immunodetection findings showed that scattered Kit-positive cells (identified as mast cells and used as an internal control) were observed in the patient's tissue, numerically distributed as in normal tissue (24.9/

mm² \pm 2.6/mm² *vs* 25.7/mm² \pm 3.8 /mm²) (Figures 1A and 1B). By contrast, in both parietal cells and intrinsic innervation locations Kit immunoreactivity was completely absent in the patient's tissue (Figures 1D and 1F), whereas in control tissues Kit was intensely expressed in 10% of mucosal parietal cells and at the level of the intrinsic innervation (1.8%) (Figures 1C and 1E). Since previous studies have shown that functional Kit-positive ICC are disappeared in the murine small bowel when Kit receptors are blocked^[24], we used a TUNEL method to determine whether ICC undergo apoptosis in gastroparesis. No evidence for apoptosis was observed (data not shown).

Intracytoplasmic expression of SCF was intense with membrane reinforcement in 70% of the patient's parietal cells and weaker with no membrane reinforcement in controls (Figures 2A and 2B), while the positivity for SCF, absent at the intrinsic innervation level, was diffuse throughout the tunica muscularis, in both gastroparesis and normal tissues. In order to make quantitative comparisons of SCF staining patterns in normal and diseases tissues, immunoreactive scores were determined as described by Remmele *et al*^[34] and subjected to statistical analysis, which showed significantly elevated levels of SCF in the patient's tissue sample (Figure 3).

Serum levels of soluble SCF

Serum levels of SCF in the patient (332.7 ng/L) were below the range detected in controls (mean: 741 ng/L, range: 558-1441 ng/L).

DISCUSSION

Little is known about the pathogenesis of gastroparesis,

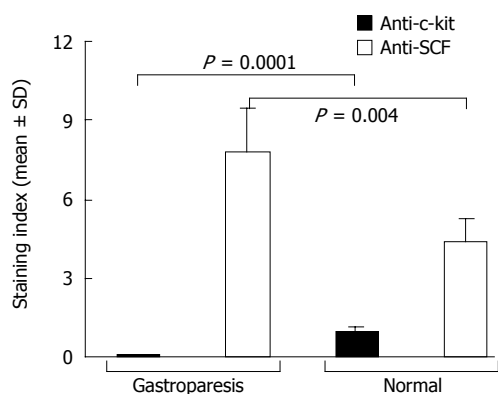


Figure 3 Quantitative evaluation of SCF expression on parietal cells in gastric tissue specimens from control subjects and the patient with gastroparesis. Differences in the immunoreactive score, obtained as described in the text, were assessed by *t*-test.

and apart from causes secondary to well-known conditions (diabetes mellitus, gastric surgery) only sporadic cases of idiopathic myenteric ganglionitis^[4] or infective causes (bacterial, viral) preceding the onset of symptoms have been identified^[35,36]. Unfortunately, pathological studies of idiopathic gastroparesis are rare, and the early ones revealed no abnormalities of the smooth muscle or the myenteric plexus^[37,38]. More recent evidence (with the introduction of Kit immunodetection) points to a reduction of gastric ICC in idiopathic gastroparesis, both in a murine model^[30] and in a single case report^[31], although the potential role in this condition of SCF, the critical factor for Kit signaling in ICC^[39], has not been investigated.

We report a case of severe gastroparesis refractory to diverse therapeutic measures, which eventually required total gastrectomy to relieve the symptoms, and in which there was complete disruption of the Kit-positive ICC network together with a reduction in number and size of ganglion cells in the submucous and myenteric plexus *vs* normal counterparts. No signs of apoptosis in the pacemaker and myenteric regions were observed, suggesting that degenerative injuries are not involved in these abnormalities. To the best of our knowledge, a total lack of detectable ICC has never been described in a “functional” gastrointestinal disorder. Since the gastric ICC network is essential for slow wave generation and plays a key role in neurotransmission of cholinergic excitation and inhibition due to nitric oxide^[17,40], this complete disruption likely explains the significant symptoms, the slow gastric emptying and the lack of response to therapeutic interventions. The lack of detectable Kit-positive ICC may be a primary event or secondary to a loss of connected signaling molecules. Previous studies have shown that ICC can be influenced to abandon the normal course of development, lose pacemaker function, and radically change its morphology if Kit is blocked or if an unsuitable form of SCF is presented^[21,41].

The binding of SCF to Kit receptors is essential to initiate a signaling pathway in ICC, which is essential for their normal development and rhythmic activity in the gastrointestinal tract^[16,42-45]. We also investigated the expression of SCF in the patient's mucosa, and found that intracytoplasmatic and membrane SCF levels were

particularly high in parietal cells *vs* controls. In addition, as in the normal counterpart, gastroparesis tissue showed diffuse positivity for SCF throughout the tunica muscularis, potentially in close proximity to ICC.

The steel locus encodes two distinct isoforms of SCF, both of which are synthesized as transmembrane proteins expressed at the cell surface^[46]. However, one isoform, known as soluble SCF, is rapidly cleaved by proteolytic processing and released, while the other, known as membrane-bound SCF isoform, lacking the proteolytic cleavage site, releases soluble SCF much more slowly^[47,48]. Soluble and membrane-bound isoforms of SCF are expressed in the gastrointestinal tract, but the role of each isoform in supporting ICC development is unknown. However, since spontaneous Steel-Dickie mutant mice that express soluble SCF exclusively do not display spontaneous, rhythmic electric slow wave activity, and ICC are not present in the myenteric plexus^[21], membrane-bound SCF more likely plays an essential role in development and/or maintenance of ICC. Since membrane-bound SCF expression was not only retained but even increased in gastric parietal cells and supporting cells potentially in close proximity to ICC in the patient's tissue specimen, we can postulate that in this case of gastroparesis a defective expression of Kit receptor by ICC rather than the absence of these cells leads to accumulation of SCF in this case of gastroparesis. Studies showing that mice harboring inactivating mutations in the *c-kit* gene lack ICC and their gastrointestinal tract fails to display any slow wave-type action potential^[16,24] are in favour of this hypothesis. Since the blocking of Kit receptors in the mouse causes transdifferentiation of ICC to a smooth muscle phenotype^[35], this inherent plasticity between ICC and smooth muscle cells might underlie this clinical condition. If verified, this hypothesis might be exploited, because if ICC do not die in gastroparesis but redifferentiate, it might be possible to create conditions that would shift the phenotype back toward ICC.

Besides the increased local accumulation of SCF in this case of gastroparesis, the soluble circulating form estimated by specific ELISA in the serum, was found to be decreased in our patient compared with healthy subjects. This decreased SCF serum concentration is difficult to explain and correlate with the disease, because several cell types, such as stromal cells, fibroblasts and endothelial cells, present in different tissues as well as in the gastrointestinal tract, express and release SCF and may contribute to the circulating soluble form. Moreover, SCF is thought to act locally, close to the site of production, where the concentration is likely to be much higher^[49].

This patient has undergone numerous treatments with several different prokinetic drugs, but none of them proved to be effective, as is often the case in this condition^[50]. Recently, electrical stimulation of enteric nerves and/or pacing of gastric slow wave activity have been attempted in gastroparesis refractory to medical treatment^[51]. Although reports appear to be encouraging (decreased frequency of vomiting and gastrointestinal symptoms, and improved quality of life)^[52], it must be kept in mind that this is still to be considered an experimental option available only in a few research centers worldwide.

Moreover, it should also take into consideration that, in some cases, pacing the stomach may not be effective in improving emptying when the primary cause of abnormal motility is due to insufficient excitation by the enteric nerves.

The above considerations also leave little room for other therapeutic options, such as injecting botulinum toxin into the pyloric sphincter^[53], and a surgical approach may be necessary, and is possible if small bowel function is intact. Only limited data on surgical treatment of gastroparesis are available, showing that complete gastrectomy may be effective for surgical gastroparesis, whereas a more cautious approach is required for the diabetic or idiopathic forms^[54].

In conclusion, we report a case of severe idiopathic gastroparesis, which was likely due to impaired gastric motility, the absence of ICC, or more likely due to their functional absence (i.e., ICC were present but not expressing Kit receptors, and therefore not functioning). Since this is the first reported abnormality, these findings may be useful for a better understanding of the pathophysiology of “idiopathic” gastroparesis and perhaps, for a more targeted therapeutic approach. Moreover, there is evidence that enteric abnormalities are also found in other so-called “functional” disorders (such as irritable bowel syndrome)^[55], thus leading to a reconsideration of the classification of these entities, in which organic abnormalities are increasingly found.

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RAPID COMMUNICATION

Polaprezinc protects human colon cells from oxidative injury induced by hydrogen peroxide: Relevant to cytoprotective heat shock proteins

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Abstract

AIM: To investigate the effect of polaprezinc on cellular damage induced by hydrogen peroxide (H_2O_2) in human colon CaCo2 cells.

METHODS: CaCo2 cells were treated with polaprezinc (10-100 $\mu\text{mol/L}$) for 6 h. After polaprezinc treatment, the cells were incubated with H_2O_2 (20 $\mu\text{mol/L}$) for 1 h. Cell viability was measured by MTT assay. Western blot analysis for heat shock protein (HSP) 27 and HSP72 in the cells was performed. Moreover, cells were pretreated with quercetin (200 $\mu\text{mol/L}$), an inhibitor of HSP synthesis, 2 h before polaprezinc treatment, and cell viability and the expression of HSP27 and 72 were assessed in these cells.

RESULTS: Polaprezinc significantly protected CaCo2 cells from cell damage induced by H_2O_2 , and up-regulated the expressions of HSP27 and HSP72 in the cells (10, 30 and 100 $\mu\text{mol/L}$ of polaprezinc; $35.0\% \pm 7.7\%$, $58.3\% \pm 14.6\%$ and $64.2\% \pm 8.2\%$, respectively. $P < 0.01$ versus polaprezinc-nontreated cells; $6.0\% \pm 4.4\%$). Quercetin inhibited the up-regulation of HSP27 and HSP72 by polaprezinc and diminished the protective effect of polaprezinc against H_2O_2 -caused injury in the cells.

CONCLUSION: Polaprezinc is a useful therapeutic agent for treatment of colitis and its effects depend on the function of cytoprotective HSP in colon.

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INTRODUCTION

Polaprezinc [N-(3-aminopropionyl)-L-histidinato zinc], an antiulcer drug, is a chelate compound consisting of zinc ion, L-carnosine, dipeptide of β -alanine, and L-histidine and has an antioxidant effect and anti-*H. pylori* activity^[1-4]. It has been reported that administration of polaprezinc prevents gastric mucosa from tissue injury in experimental models^[5-9]. Additionally, recent works indicate that polaprezinc has a therapeutic effect in two models of experimental colitis^[10,11]. On the other hand, some studies have shown that polaprezinc up-regulates the expression of heat shock protein (HSP) in stomach and colon^[11,12].

HSP, a highly conserved and ubiquitous protein, is up-regulated to protect against various physiological stress conditions such as infection and ischemia^[13]. Some HSPs are now accepted to be key anti-inflammatory molecules and play an important role in the protection against physiologic and environmental stressors^[14]. Overexpression of these HSPs are thought to prevent apoptosis by regulating intracellular intermediates intimately involved in apoptotic signaling. In intestine, up-regulation of these HSPs by chemicals or non-lethal thermal stress has been shown to protect intestinal epithelial cells and colon tissues against injurious stimulants *in vitro* and *in vivo*^[15-20]. In particular, HSP27 and HSP72 protect cells and tissues from chemical, infectious and ischemic injury^[13]. On the other hand, our previous study demonstrated that polaprezinc up-regulates the expression of HSP27 and HSP72 in the mouse colon^[11], but there are no investigations of the effect of polaprezinc on HSP expression in intestinal epithelial cells *in vitro*. Thus, we herein assessed the effect of polaprezinc on cell damage induced by oxidative stress, which often occurs in intestine

and injured colonic epithelial cells *in vitro*. Moreover, we investigated whether the protective effect of polaprezinc depends on the function of HSPs in human colon cells.

MATERIALS AND METHODS

Materials

Polaprezinc was a gift from Zeria Pharmaceuticals Co. (Saitama, Japan). Antibodies against HSP25 and HSP72 were obtained from Stressgen (Victoria, BC, Canada). Nitrocellulose membrane filters were from Millipore (Bedford, MA). An ECL Western blotting detection system kit was from Amersham Bioscience (Piscataway, NJ, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody and a Micro BCA protein assay kit were from Pierce (Rockford, IL, USA). Quercetin was from Wako Pure Chemical Industries (Osaka, Japan). A CellTiter 96 aqueous one solution cell proliferation assay kit was from Promega (Madison, WI, USA) and an anti-actin antibody was from Sigma-Aldrich Co. (Temecula CA, USA). All other chemicals were of reagent grade.

Cell culture and treatment with polaprezinc

CaCo2 cells (between passages 8 and 14) were grown in high glucose Dulbecco's Vogt modified Eagle's media (DMEM, Sigma, St Louis, MO, USA) supplemented with 100 mL/L fetal calf serum. Cells were incubated at 37°C in 50 mL/L CO₂ and 90% humidity. Each experiment was performed with an 80%-90% confluent monolayer. Polaprezinc was diluted in DMEM and added to the cultured cells at a final concentration of 10-100 µmol/L for 6 h.

Examination of cell viability

To examine the protective effect of polaprezinc on CaCo2 cells against oxidant-induced injury, we assessed cell viability by MTT assay as described previously^[20]. Briefly, cells were grown to an approximate cell concentration of 10⁴ cells/well in 96-well plates. Cells in each well were incubated at 37°C with 150 µL of DMEM and 50 µL of CellTiter 96 aqueous, one solution cell proliferation reagent. Cell viability was determined by the generation of a formazan dye from the substrate. Absorbance (*A*) at 490 nm was measured with a spectrometer 0 and 90 min after addition of CellTiter 96 aqueous, one solution cell proliferation reagent. Difference in *A* between 0 and 90 min after incubation was calculated for evaluation of cell viability. The assay was run on cells without treatment as a negative control. The results were compared to those in a negative control and expressed as percentage of cell viability. All experiments were repeated more than three times to confirm reproducibility.

Western blot analysis

Cells were collected with 1 g/L trypsin and homogenated with a Polytron homogenizer (Kinematica, Lucerne, Switzerland). Equal amounts of homogenates were dissolved in 20 µL of Tris-HCL, 50 mmol/L (pH 6.8), containing 10 g/L 2-mercaptoethanol, 20 g/L SDS, 200 mL/L glycerol and 0.4 g/L bromophenol blue. The samples were heated at 100°C for 5 min, then subjected to SDS-polyacrylamide

gel electrophoresis (SDS-PAGE) and transferred electrophoretically onto a nitrocellulose membrane. The membranes were blocked with 10 mL/L nonfat dry milk in PBS, probed with HSP 27 and 72 Ab, and reacted with goat anti-rabbit IgG Ab coupled with horseradish peroxidase (HRP). The resultant complexes were processed for the ECL detection system according to the manufacturer's protocol. Protein concentration in the homogenate was quantified using a Micro BCA protein assay reagent kit (Pierce, Rockford, IL, USA).

Quercetin treatment

Quercetin is known to strongly inhibit the HSP synthesis^[15,20,21]. To investigate the effect of quercetin, cells were pretreated with quercetin at a final concentration of 200 µmol/L for 2 h before polaprezinc treatment. Cell viability and expression of HSP27 and HSP72 were assessed in the cells treated with quercetin.

Statistical analysis

Data obtained by MTT assay were presented as mean ± SD and statistically analyzed using an analysis of variance (ANOVA), followed by Turkey's comparison test (Stat View, SAS Institute, Cary, NC). *P* < 0.05 was considered statistically significant.

RESULTS

Polaprezinc protected CaCo2 cells against hydrogen peroxide

We assessed the effect of polaprezinc on oxidative injury in the CaCo2 cells. Morphological alteration and growth inhibition were not observed in the CaCo2 cells after exposure to polaprezinc (data not shown). To evaluate the effect of polaprezinc on oxidative stress, cell viability in CaCo2 cells treated with NH₂Cl was analyzed by MTT assay. MTT assay showed that, the difference in *A* at 490 nm, as a parameter of cell viability, was significantly decreased in the cells treated with hydrogen peroxide at a final concentration of 20 mol/L (6.0% ± 4.4%). In contrast, we found that 10, 30 and 100 µmol/L of polaprezinc (35.0% ± 7.7%, 58.3% ± 14.6% and 64.2% ± 8.2%, respectively) significantly improved viability in the cells at 6 h after polaprezinc treatment compared with the cells without polaprezinc treatment (*P* < 0.01).

Enhancement of HSP27 and HSP72 expression in CaCo2 cells treated with polaprezinc

For assessment of the HSP expression in the CaCo2 cells treated with polaprezinc, Western blot analysis for HSPs in the cells was carried out. It was found that HSP27 and HSP72 were constitutively expressed in the CaCo2 cells without treatment. The expressions of HSP25 and HSP72 was greatly up-regulated in the CaCo2 cells treated with 10, 30 and 100 µmol/L of polaprezinc for 6 h compared with those in the non-treated CaCo2 cells (Figure 1).

Quercetin inhibited up-regulation of HSP27 and 72 and diminished the protective effect of polaprezinc on oxidative injury in Caco2 cells

To clarify the effect of HSPs induced by polaprezinc

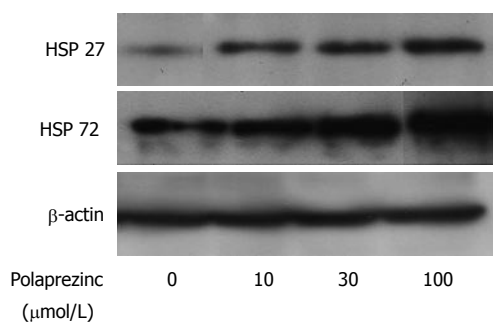


Figure 1 Effect of polaprezinc on up-regulation of heat shock protein HSP27 and HSP70 in CaCo2 cells. The expression of HSP27 and HSP72 was markedly up-regulated in the CaCo2 cells treated with polaprezinc. Representative results of three complete experiments are shown.

on cell injury, we investigated the effect of quercetin, an inhibitor of HSP synthesis, and polaprezinc on CaCo2 cells injured by H_2O_2 . Pretreatment with 200 $\mu\text{mol/L}$ of quercetin for 2 h considerably reduced the cell viability in the CaCo2 cells treated with 30 $\mu\text{mol/L}$ of polaprezinc compared with the cells without quercetin treatment when cells were exposed to H_2O_2 ($P < 0.01$; $12.0\% \pm 0.3\%$ and $53.6\% \pm 1.9\%$, respectively), whereas the cell viability in the quercetin-treated CaCo2 cells without H_2O_2 was minimal compared with non-treated cells ($94.3\% \pm 0.1\%$ and $98.8\% \pm 0.1\%$, respectively). In HSP expression, 200 $\mu\text{mol/L}$ of quercetin completely inhibited the up-regulation of HSP27 and HSP72 by polaprezinc in the CaCo2 cells (Figure 2). On the other hand, quercetin did not alter the baseline level of HSP27 and HSP72 in the cells without polaprezinc treatment (Figure 2).

DISCUSSION

In this study, we demonstrated that polaprezinc improved cell viability of CaCo2 cells injured by oxidative chemicals. Moreover, polaprezinc remarkably up-regulated the expression of HSP27 and HSP72, which play an important role in protecting the cells from stresses. Additionally, quercetin, an inhibitor of HSP synthesis diminished the protective effect of polaprezinc on cell injury by H_2O_2 and the up-regulation of HSP27 and HSP72 in CaCo2 cells. These results are consistent with our previous observation that polaprezinc can up-regulate the expressions of HSP27 and HSP70 in murine experimental colitis^[11]. Recent studies have demonstrated that the expression of HSP27 and HSP70 is up-regulated by mild physiological stress and irritants^[13,14]. Furthermore, HSPs have been suggested to be key anti-inflammatory molecules and play a critical role in protective mechanism against severe physiologic and environmental stressors^[13,14]. High expression levels of HSPs are thought to prevent apoptosis by regulating intracellular intermediates intimately involved in apoptotic signaling.

In intestine, induction of HSPs by chemicals or non-lethal thermal stress has been shown to protect intestinal cells and colon tissues against injury and damage^[15-19]. Musch *et al*^[15] reported that induction of HSP72 by

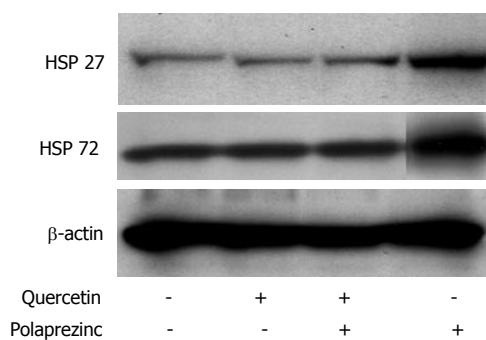


Figure 2 Effect of quercetin on induction of HSP27 and HSP72 in CaCo-2 cells treated with polaprezinc. Quercetin did not up-regulate the expression of HSP27 and HSP72 in the cells. Representative results of three complete experiments are shown.

hyperthermia protects rat intestinal epithelial cells from oxidative injury. Hyperthermia rapidly and reproducibly induces HSPs in intestinal epithelial cells. Otani *et al*^[16] demonstrated that preinduction of HSPs by non-lethal hyperthermia protects rats from colitis induced by acetic acid. Hyperthermia elevates the expression of HSPs, including HSP72, and remarkably reduces the severity of acetic acid-induced colitis in the colons of rats, suggesting that HSPs are important for the protection against colon tissue damage such as colitis. Although numerous studies have clarified the protecting effect of polaprezinc, there are a few investigations on the up-regulation of HSP by polaprezinc. Very recently, it has been reported that polaprezinc up-regulates the expression of HSP72 in cultured rat gastric mucosal cells (RGM1) and rat gastric mucosa^[12]. In colon, our previous study demonstrated that polaprezinc enhances the up-regulation of HSP72 in mouse colon during colitis^[11]. However, these studies have not fully clarified the role of HSP induced by polaprezinc. In this study, we demonstrated that quercetin, an inhibitor of HSP synthesis, diminished the protective effect of polaprezinc on cell damage induced by H_2O_2 and up-regulated HSP, suggesting that polaprezinc-induced HSP is essential for protection against oxidative injury in colon cells.

Besides HSP70, HSP27, a member of the small HSP family, have also been found to play an important role in protection of cells against stresses^[13]. Ropeleski *et al*^[18] demonstrated that IL-11-induced HSP27 has a protective effect against oxidative stress induced by monochloramine in cultured rat intestinal epithelial cells, as HSP27 plays a potential role in cytoprotection in intestine. Consistent with these findings and our previous findings, the expression of HSP27 is markedly up-regulated in the CaCo2 cells treated with polaprezinc. However, quercetin did not up-regulate the expression of HSP27 in CaCo2 cells treated with polaprezinc in our study. These findings provide the idea that the protective effect of polaprezinc depends not only on the function of HSP72 but also on HSP27 in colon cells.

The mechanism by which polaprezinc up-regulates the expression of HSPs has not been precisely elucidated. Interestingly, it has been reported that zinc or L-carnitine

cannot up-regulate the expression of HSPs in gastric epithelial cells and rat gastric mucosa^[12]. Our previous study indicates that zinc supplement does not induce HSPs in mouse colon^[11]. Although we did not show the effects of zinc or L-carnocine on induction of HSPs in colon cells, polaprezinc may be a powerful inducer of HSPs compared with zinc or L-carnocine. The reason why polaprezinc but not zinc or L-carnocine markedly up-regulates the expression of HSPs is unclear, thus, further study is needed to clarify this result.

In conclusion, polaprezinc protects colon cells from oxidative injury induced by hydrogen peroxide, and enhances the expression of HSP27 and HSP72 in the CaCo2 cells. The up-regulation of cytoprotective HSPs by polaprezinc is potentially therapeutic for intestinal injuries such as colitis.

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RAPID COMMUNICATION

Expression of vascular endothelial growth factor and its receptors VEGFR-1 and 2 in gastrointestinal stromal tumors, leiomyomas and schwannomas

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Abstract

AIM: To investigate the role of vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 and 2 in the growth and differentiation of gastrointestinal stromal tumors (GISTs).

METHODS: Thirty-three GISTs, 15 leiomyomas and 6 schwannomas were examined by immunohistochemistry in this study.

RESULTS: VEGF protein was expressed in the cytoplasm of tumor cells, and VEGFR-1 and 2 were expressed both in the cytoplasm and on the membrane of all tumors. Immunohistochemical staining revealed that 26 GISTs (78.8%), 9 leiomyomas (60.0%) and 3 schwannomas (50.0%) were positive for VEGF; 24 GISTs (72.7%), 12 leiomyomas (80.0%) and 4 schwannomas (66.7%) were positive for VEGFR-1; 30 GISTs (90.9%), 5 leiomyomas (33.3%) and 4 schwannomas (66.7%) were positive for VEGFR-2. VEGFR-2 expression was statistically different between GISTs and leiomyomas ($P < 0.0001$). However, there was no correlation between the expression of VEGF pathway components and the clinical risk categories.

CONCLUSION: Our results suggest that the VEGF pathway may play an important role in the differentiation of GISTs, leiomyomas and schwannomas.

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Key words: Gastrointestinal stromal tumor; Leiomyoma; Schwannoma; Vascular endothelial growth factor; Vascular endothelial growth factor receptors

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are rare mesenchymal tumors of the gastrointestinal (GI) tract that may occur from the oesophagus to the anus, including the omentum^[1,2]. Despite their rarity, GISTs are the most common primary mesenchymal tumors of the GI tract^[1-3]. The mechanisms of tumorigenesis, progression and differentiation of GISTs are unknown. Traditionally, all primary mesenchymal spindle cell tumors of the GI tract are uniformly classified as smooth muscle tumors (e.g., leiomyomas, cellular leiomyomas or leiomyosarcomas). Tumors with epithelioid cytologic features are designated leiomyoblastomas or epithelioid leiomyosarcomas^[4]. Recently, Sircar *et al*^[5] postulated that GISTs originate from Cajal cells in the GI tract and differ from leiomyomas and schwannomas, which are of mesenchymal cell origin. Cajal cells are thought to be gastrointestinal pacemaker cells that regulate intestinal motility^[6]. GISTs are characterized by frequent expression of the bone marrow leukocytic progenitor cell antigen CD34^[7] and the c-kit proto-oncogene^[2,3,5].

Some GISTs have mutations in the genes encoding c-kit and platelet-derived growth factor alpha (PDGFR- α) that cause constitutive tyrosine kinase activation^[3,8-10]. Tumors expressing c-kit or PDGFR- α oncoproteins are indistinguishable with respect to activation of downstream signaling intermediates and cytogenetic changes associated with tumor progression. C-kit and PDGFR- α mutations appear to be alternative and mutually exclusive oncogenic mechanisms in GISTs^[9,10].

Vascular endothelial growth factor (VEGF) has been identified as a key regulator of tumor angiogenesis, and VEGF receptors (VEGFR) are the major mediators of the mitogenic and permeability-enhancing effects of VEGF in endothelial cells^[11,12]. In addition, VEGF is a survival factor for endothelial cells, and a marked dependence on VEGF has been shown in newly formed but not established

tumor vessels^[13]. Although the field of tumor angiogenesis is an area of extensive research, the consequences of enhanced angiogenesis and its reversion on tumor growth and progression are only partially elucidated^[14-16]. Recently, coexpression of VEGF and its receptor, either VEGFR-1 (Flt-1) or VEGFR-2 (Flk-1/KDR), has been reported in tumor cells, suggesting the presence of an autocrine and/or a paracrine VEGF/VEGFR growth pathway in solid tumors^[17-19]. Further, the expression levels of VEGF and its receptors have been shown to correlate with progressive tumor growth and development of metastasis by many carcinomas^[20].

These studies suggest that the VEGF pathway is involved in tumor growth and differentiation. However, there are no data detailing VEGFR expression in GISTs, leiomyomas or schwannomas, or the role of VEGF in the etiology of these tumors. The purpose of this study was to investigate the expression of VEGF and VEGFRs in GISTs.

MATERIALS AND METHODS

Samples

Thirty-three specimens of GISTs (28 from the stomach and 5 from the small intestine), 15 specimens of leiomyomas (4 from the oesophagus, 4 from the stomach and 7 from the large intestine), 6 specimens of schwannomas (5 from the stomach and 1 from the large intestine) were selected from surgical pathology archival tissues at Nagasaki University Hospital between 2001 and 2006. The specimens of GISTs, leiomyomas and schwannomas were 0.8-12.0 cm, 0.1-4.5 cm and 0.6-5.0 cm, respectively. In this study, GISTs were defined as expressing both c-kit and CD34 surface antigens and classified by risk categories, mitosis counts and tumor size^[21]. The number of mitoses was determined by counting 50 high-power fields (HPF, $\times 400$) under a Nikon (Tokyo, Japan) E400 microscope. Leiomyomas were defined both as expressing α -smooth muscle cell actin (SMA) but not c-kit, CD34 or S100-protein and as expressing S100-protein but not c-kit, CD34 or SMA. Two independent pathologists (T. Nakayama and I. Sekine) determined tumor identification/classification.

Immunohistochemical staining

The subcellular localization of VEGF, VEGFR-1 and 2 was determined in GISTs using polyclonal antibodies directed against unique sequences of VEGF, VEGFR-1 and 2. These antibodies were devoid of any cross-reaction with other proteins in the VEGF family. Formalin-fixed and paraffin-embedded specimens were cut into 4 μ m thick sections, deparaffinized and preincubated with normal bovine serum to prevent non-specific binding. The sections were incubated overnight at 4°C with primary polyclonal antibody to human VEGF [(147), 1 mg/L; Santa Cruz Biotechnology Inc., Santa Cruz, CA], VEGFR-1 [Flt-1(C-17), 1 mg/L; Santa Cruz Biotechnology Inc.] or VEGFR-2 [Flk-1(C-20), 1 mg/L; Santa Cruz Biotechnology Inc.], followed by alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (0.4 μ g/mL; Santa Cruz Biotechnology, Inc.). The reaction products

Table 1 Immunohistochemistry of VEGF pathway components in intestinal stromal tumours, *n* (%)

	<i>n</i>	VEGF		VEGFR-1		VEGFR-2	
		-	+	-	+	-	+
GISTs	33	7 (21.2)	26 (78.8)	9 (27.3)	24 (72.7)	3 (9.1)	30 (90.9) ^b
Leiomyoma	15	6 (40.0)	9 (60.0)	3 (20.0)	12 (80.0)	10 (66.7)	5 (33.3)
Schwannoma	6	3 (50.0)	3 (50.0)	2 (33.3)	4 (66.7)	2 (33.3)	4 (66.7)

^b*P* < 0.0001 vs leiomyomas.

were visualized using a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride (BCIP/NBT; Roche Diagnostic Corp., Indianapolis, IN). Negative controls replaced the primary antibody with non-immunized rabbit serum, and human breast cancer tissue served as the positive control^[22]. VEGF, VEGFR-1 and 2 expressions were classified into 3 categories depending upon the percentage of cells stained and/or the intensity of staining (-: 0% to 15% positive tumor cells; +: > 15% positive tumor cells).

Statistical analysis

The Stat View II program (Abacus Concepts, Inc., Berkeley, CA) was used for statistical analysis. Analyses comparing the degree of VEGF, VEGFR-1 or 2 expressions in GISTs, leiomyomas and schwannomas were performed using the Mann-Whitney's test.

RESULTS

The results of immunohistochemical stainings for VEGF, VEGFR-1 or 2 are summarized in Table 1. VEGF, VEGFR-1 and 2 expression was heterogeneous in GISTs, leiomyomas and schwannomas and localized to the cytoplasm and/or membrane of tumor cells (Figure 1). Immunohistochemical staining revealed VEGF expression in the cytoplasm of GIST (Figure 1A), leiomyoma (Figure 1B) and schwannoma (Figure 1C) cells. VEGFR-1 expression was shown in the membrane and cytoplasm of GIST (Figure 1E), leiomyoma (Figure 1F) and schwannoma (Figure 1G) cells. VEGFR-2 was expressed in the membrane and cytoplasm of GIST (Figure 1I), leiomyoma (Figure 1J) and schwannoma (Figure 1K) cells. Immunohistochemical staining was positive for VEGF in 26 (78.8%) of 33 GISTs, 9 (60.0%) of 15 leiomyomas and 3 (50.0%) of 6 schwannomas, respectively. Twenty-four (72.7%) of GISTs, 12 (80.0%) of leiomyomas and 4 (66.7%) of schwannomas showed positive staining for VEGFR-1. There was no statistical difference in VEGF or VEGFR-1 expression between GISTs and leiomyomas or schwannomas. Immunohistochemical staining was positive for VEGFR-2 in 30 (90.9%) of GISTs, 5 (33.3%) of 15 leiomyomas and 4 (66.7%) of schwannomas. There was a statistical difference in VEGFR-2 expression between GISTs and leiomyomas (*P* < 0.0001).

The classification of GISTs by risk category, mitosis counts and tumor size is shown in Table 2. All 4 cases within the high risk category expressed VEGF, VEGFR-1 and 2. All 4 cases with over 10 mitoses per 50 HPFs strongly expressed VEGF, VEGFR-1 and 2. Finally, only one tumor that measured over 10 cm strongly

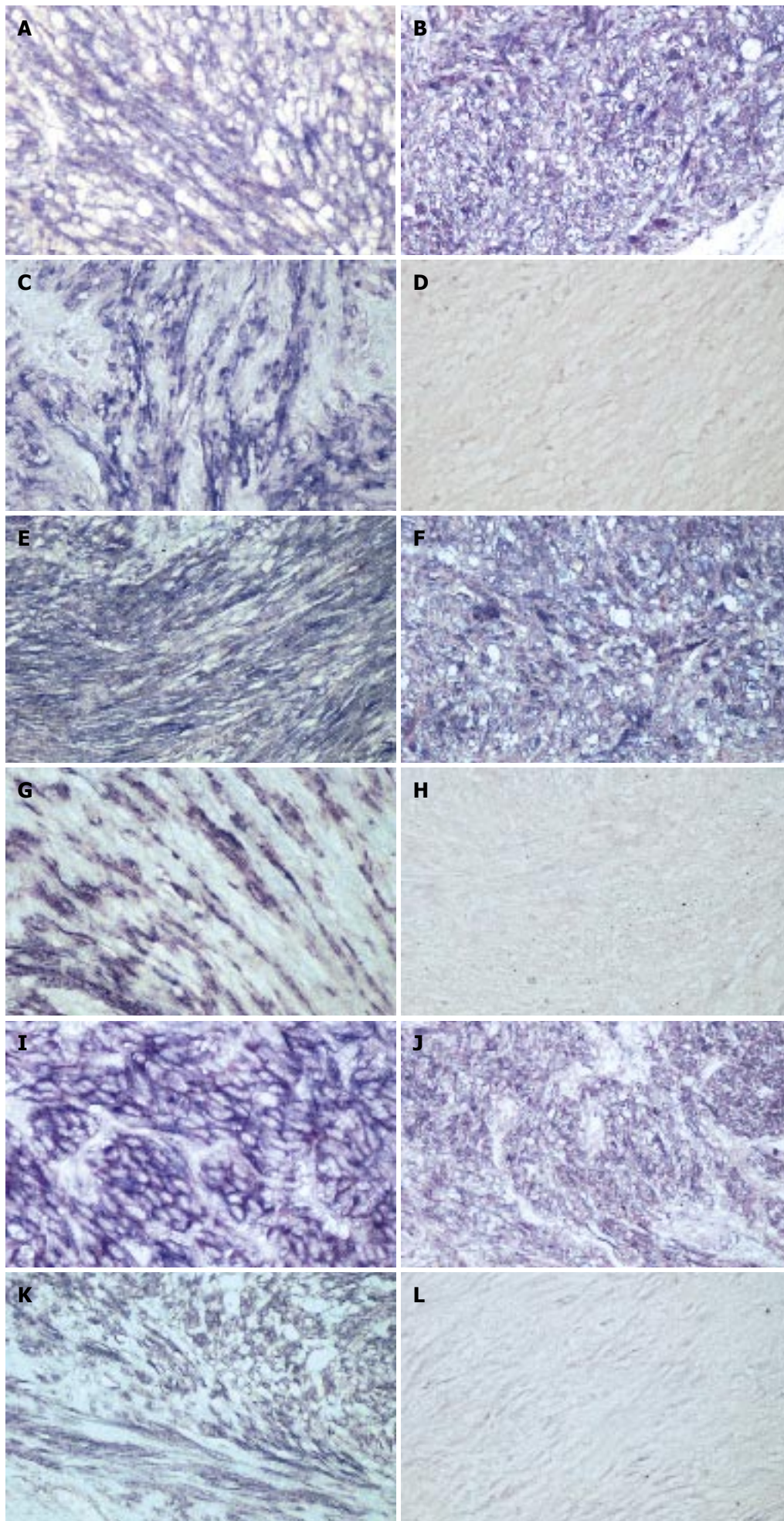


Figure 1 Immunohistochemical staining reveals VEGF expression in the cytoplasm of GIST (A), leiomyoma (B) and schwannoma (C) cells; VEGFR-1 expression in the membrane and cytoplasm of GIST (E), leiomyoma (F) and schwannoma (G) cells; VEGFR-2 expression in the membrane and cytoplasm of GIST (I), leiomyoma (J) and schwannoma (K) cells; negative staining of GIST for VEGF, VEGFR-1 or VEGFR-2 in Figure D, H or L, respectively. BCIP/NBT reaction product demonstrating VEGF, VEGFR-1 and 2 levels. (magnification: x 200).

Table 2 VEGF, VEGFR-1 and 2 expression and categories for GISTs (*n* = 33) *n* (%)

Total	<i>n</i> 33	VEGF		VEGFR-1		VEGFR-2	
		-	+	-	+	-	+
		7	26	9	24	3	30
Risk categories		NS		NS		NS	
High	4	0 (0.0)	4 (100)	0 (0.0)	4 (100)	0 (0.0)	4 (100)
Intermediate	5	1 (20.0)	4 (80.0)	2 (40.0)	3 (60.0)	0 (0.0)	5 (100)
Low	17	3 (17.6)	14 (82.4)	5 (29.4)	12 (70.6)	2 (11.8)	15 (88.2)
Very low	7	3 (42.9)	4 (57.1)	2 (28.6)	5 (71.4)	1 (14.3)	6 (85.7)
Mitosis counts (per 50 fields, HPF)		NS		NS		NS	
< 2	17	4 (23.5)	13 (76.5)	6 (35.3)	11 (64.7)	2 (11.8)	15 (88.2)
2-5	8	2 (25.0)	6 (75.0)	3 (37.5)	5 (62.5)	1 (12.5)	7 (87.5)
6-10	4	1 (25.0)	3 (75.0)	0 (0.0)	4 (100)	0 (0.0)	4 (100)
>10	4	0 (0.0)	4 (100)	0 (0.0)	4 (100)	0 (0.0)	4 (100)
Tumour size (cm in length)		NS		NS		NS	
< 2	6	3 (50.0)	3 (50.0)	2 (33.3)	4 (66.7)	1 (16.7)	5 (83.3)
2-< 5	20	3 (15.0)	17 (85.0)	5 (25.0)	15 (75.0)	2 (10.0)	18 (90.0)
5-< 10	6	1 (16.7)	5 (83.3)	2 (33.3)	4 (66.7)	0 (0.0)	6 (100)
> 10	1	0 (0.0)	1 (100)	0 (0.0)	1 (100)	0 (0.0)	1 (100)

NS: Not significant.

expressed VEGF, VEGFR-1 and 2. However, there was no correlation between VEGF, VEGFR-1 or VEGFR-2 expression and each classification.

DISCUSSION

The coexpression of VEGF and VEGFR-1 and 2 has been reported in tumor cells, suggesting the presence of an autocrine and/or a paracrine VEGF/VEGFR growth pathway in solid tumors^[17-19]. VEGF also has been shown to play a role in the proliferation and/or differentiation of stromal tumors and normal mesenchymal cells^[23-26]. VEGF expression in GISTs has been already reported^[27,28]. However, there are no studies on VEGF receptor expression in GISTs, leiomyomas and schwannomas, or on the potential roles of VEGF and its receptors in the growth of these tumors. This is the first study to determine the expression of VEGF receptors in GIST and stromal tumors, demonstrating substantial levels of VEGF and its receptors in the cytoplasm of GIST, leiomyoma and schwannoma cells. Therefore, we suggest that VEGF and its receptors may play an important role in the growth and/or differentiation of intestinal stromal tumors via autocrine and/or paracrine pathways.

We did not find any statistical correlation between risk grade and the expression of VEGF or VEGFRs for GISTs. However, all 4 GISTs in the high risk category expressed VEGF and VEGFRs (Table 2). Furthermore, all 4 GISTs that had higher mitosis counts (over ten per 50 HPFs) were positive for VEGF and VEGFRs. Our data suggest that high risk GIST- expressed VEGF and VEGFR level is higher than normal. We thought that the number of high risk GISTs should be less. Further studies are needed to examine the VEGF/VEGFR pathway components in high risk GISTs.

VEGF induces a variety of enzymes and proteins important in the degradation process, including matrix-degrading metalloproteinase, interstitial collagenase, and serine proteases such as urokinase-type plasminogen

activator (u-PA) and tissue-type plasminogen activator (t-PA)^[29,30]. In this study, we did not evaluate the invasive activities of GIST cells, because all the GISTs were solitary and showed clear margins. However, the activation of these factors by VEGFRs provides for the possibility of conduction to a prodegradative environment that facilitates migration and invasion of tumor cells.

Solid tumors develop regions of low oxygen tension because of an imbalance in oxygen supply and consumption. Hypoxia in the tumor microenvironment is sufficient to activate hypoxia-inducible factor (HIF)-dependent gene expression^[31]. HIF-1 alpha (HIF-1 α) is overexpressed in most human malignancies^[32]. HIF-1 binds to HIF responsive elements in the promoter region of certain genes, such as VEGF, to increase transcription^[33]. HIF-1 expression in GIST has been already reported and suggested to contribute to tumor angiogenesis in GIST^[28]. HIF-1 might play a role in the growth of GIST, because VEGF was expressed greater in larger GISTs in this study. However, we do not have any data about hypoxia, angiogenesis or HIF-1 expression in GIST, because the purpose of this study was to clarify the role of VEGF/VEGFR pathway in GIST cells. We hope that the relationship between hypoxia and GIST growth can be clarified in next study.

Joensuu *et al.*^[34] have reported a patient in whom Imatinib (STI-571, Gleevec), a tyrosine kinase inhibitor, is effective against GIST. Imatinib has proven to be remarkably efficacious in heavily pretreated GIST patients with advanced disease in phase III clinical trials^[35]. It was reported that Imatinib down-regulates VEGF expression in the GIST cell line GIST-T1^[24]. Furthermore, anti-VEGF reagents are used in clinical trials for the therapy of colorectal, lung and breast cancer^[36]. Stimulation of VEGFR upregulates the mitogen-activated protein kinase pathway through the activation of tyrosine kinases^[37,38], the same pathway utilized by c-kit activation. These anti-VEGF reagents might be useful for the therapy of GISTs *via* the down-regulation of the VEGF/VEGFR pathway.

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RAPID COMMUNICATION

Human papillomavirus in esophageal squamous cell carcinoma in Colombia and Chile

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Abstract

AIM: To examine the presence of human papillomavirus (HPV) in esophageal squamous cell carcinoma (ESCC) specimens collected from Colombia and Chile located in the northern and southern ends of the continent, respectively.

METHODS: We examined 47 and 26 formalin-fixed and paraffin-embedded ESCC specimens from Colombia and Chile, respectively. HPV was detected using GP5+/GP6+ primer pair for PCR, and confirmed by Southern blot analysis. Sequencing analysis of L1 region fragment was used to identify HPV genotype. In addition, P16^{INK4A} protein immunostaining of all the specimens was conducted.

RESULTS: HPV was detected in 21 ESCC specimens (29%). Sequencing analysis of L1 region fragment identified HPV-16 genome in 6 Colombian cases

(13%) and in 5 Chilean cases (19%). HPV-18 was detected in 10 cases (21%) in Colombia but not in any Chilean case. Since Chilean ESCC cases had a higher prevalence of HPV-16 (without statistical significance), but a significantly lower prevalence of HPV-18 than in Colombian cases ($P = 0.011$) even though the two countries have similar ESCC incidence rates, the frequency of HPV-related ESCC may not be strongly affected by risk factors affecting the incidence of ESCC. HPV-16 genome was more frequently detected in p16 positive carcinomas, although the difference was not statistically significant. HPV-18 detection rate did not show any association with p16 expression. Well-differentiated tumors tended to have either HPV-16 or HPV-18 but the association was not statistically significant. HPV genotypes other than HPV-16 or 18 were not detected in either country.

CONCLUSION: HPV-16 and HPV-18 genotypes can be found in ESCC specimens collected from two South American countries. Further studies on the relationship between HPV-16 presence and p16 expression in ESCC would aid understanding of the mechanism underlying the presence of HPV in ESCC.

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Key words: Human papillomavirus; Esophageal squamous cell cancer; Colombia; Chile

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INTRODUCTION

Human papillomavirus (HPV), a double-stranded DNA virus, is recognized as an etiologic agent of cervical cancer^[1]. In addition, HPV is suspected of causing extragenital cancers, including cancers of the oral cavity, larynx, esophagus, and lung^[2]. However, the role of HPV

infection in the pathogenesis of those malignancies is still controversial. The relationship between HPV and esophageal squamous cell carcinoma (ESCC) has been suspected since the initial reports by Syrjanen *et al* in 1982^[3,4]. An extensive review by Syrjanen published in 2002 reported that HPV is positive in 22.9% of 1485 ESCC cases analyzed by *in situ* hybridization (ISH) and in 15.2% of 2020 ESCC cases analyzed by PCR^[5]. HPV signals were reported to present in normal cells^[6], hyperplastic and dysplastic epithelia surrounding ESCC^[7,8]. Zhou *et al*^[6] reported that HPV expression in normal epithelia rarely accompanies the expression of HPV E6 protein, whereas its expression is frequently observed in carcinoma cells. These findings suggest that the involvement of HPV may take place in very early stages of esophageal carcinomas, which is suspected of being a progressive multistage process^[9]. As postulated in development of head and neck tumors^[10], esophageal cancer may arise from the "condemned mucosa"^[11], and its development is considered to involve various risk factors, including HPV.

More than 99% of cervical cancers are known to be related to HPV^[1]. One of the interesting features of cervical cancer is marked overexpression of p16^{INK4A} (p16), a tumor suppressor which is the cyclin-dependent kinase inhibitor^[12]. P16 binds to the complex of cyclin D1 and cyclin-dependent kinase 4 and represses its ability to phosphorylate the retinoblastoma protein (pRb)^[13,14]. Hyperphosphorylation of pRb promotes unbinding of pRb and E2F while the released E2F, a well-known universal transcriptional activator, stimulates p16 transcription^[15]. At the same time, however, the free pRb negatively regulates p16 expression. In the presence of HPV, this negative feedback loop between pRb and p16 is disrupted by binding of HPV E7 to pRb since E7-bound pRb cannot regulate p16 expression. As a consequence, p16 overexpression is induced by the presence of HPV^[15]. Therefore, the comparison of p16 expression in HPV-positive and -negative ESCC may give some insight into the etiological role of HPV in ESCC development.

Although seroepidemiological evidence is important in evaluating the etiological role of HPV in ESCC, studies have reported inconsistent results. A study in Finland showed that ESCC patients have elevated serum antibody against HPV-16 when compared to blood bank donors^[16]. Whereas a study in Norway has confirmed the Finnish study^[17], a study in Sweden could not confirm it^[18]. On the other hand, a study conducted in Shaanxi Province, China, where esophageal cancer risk is known to be high, ESCC patients have elevated serum antibodies against HPV-16^[19]. However, none of these studies has tried to confirm the presence of HPV in esophageal carcinoma cells.

HPV has been found in variable proportions of ESCC, depending on the methodology and study areas^[5]. Interestingly, studies from Asian countries, especially from China, have reported relatively high percentages of HPV-positive ESCC cases when compared to reports from Western European countries. To our knowledge, there is no study comparing the HPV infection rates in the South American Continent. In the present study, we examined ESCC in Colombia and Chile, located in the northern

and southern ends of the South American Continent, and compared the detection rate of HPV in ESCC in the two countries. In addition, the association of HPV presence with p16 expression was also examined in order to evaluate the possible etiological role of HPV in ESCC development.

MATERIALS AND METHODS

Patients

We examined 47 formalin-fixed and paraffin-embedded ESCC specimens from Hospital Universitario del Valle in Cali, Colombia, from 1996 to 2001, and 26 paraffin-embedded ESCC specimens from Hospital San Camilo, San Felipe, Chile, in 1996-2000. Institutional Review Board of the Faculty of Medicine, Kagoshima University, Japan, approved the present study.

DNA extraction

Each formalin-fixed and paraffin-embedded sample was cut into 10- μ m thick sections. The specimens were treated with 1 mL of xylene and then 1 mL of ethanol. After centrifugation, the pellet was resuspended in digestion buffer (50 mmol/L Tris-Cl pH 8.0, 1 mmol/L EDTA pH 8.0, 0.5% Tween 20) containing 200 μ g of proteinase K (Invitrogen) and incubated at 56°C for 24 h. After incubation, the solution was heated at 100°C for 10 min and centrifuged. An aliquot of the supernatant was directly used for PCR.

PCR, Southern blot hybridization and sequencing

HPV amplification with GP5+/GP6+ primer pair^[20,21] was made in a reaction mix containing 2.5 μ L of template DNA, 200- μ mol/L dNTP, 0.5 μ mol/L of each primer and 1.0 U Taq DNA polymerase (Takara, Japan) in a total volume of 25 μ L reaction buffer (50 mmol/L KCl, 20 mmol/L Tris-Cl, pH 8.3). The conditions of amplification were as follows: an initial denaturation at 95°C for 4 min followed by 45 cycles at 95°C for 1 min, at 40°C for 2 min, at 72°C for 1.5 min, and a final extension at 72°C for 5 min. Beta-globin amplification with PCO3/PCO4 (110 bp) primers was used as the internal positive control. PCR conditions were as follows: an initial denaturation at 95°C for 4 min followed by 40 cycles with the cycling profile at 95°C for 1 min, at 52°C for 1 min, at 72°C for 2 min, and a final extension at 72°C for 5 min. DNA purified from Hela cells containing HPV-18 was used as external positive control.

The amplified products were revealed by electrophoresis with 3.0% agarose gels at 100 volts for 30 min. After electrophoresis, the DNA was transferred onto Hybond N+ nylon transfer membrane (Amersham, UK) by capillary blotting using 0.4 N NaOH. The generic GP5+/GP6+ PCR products amplified from HPV-18, -16, -11 and -6 were purified from agarose gels with QIAEX II extraction kits (Qiagen GmbH and Qiagen Inc., Hilden, Germany) and used as probes. The hybridization was performed at 42°C overnight and then the membranes were washed at 42°C with solution containing 6 mol/L urea, 0.4% SDS and 0.5 \times SSC buffer. For the detection of the HPV band, hybridization was carried out using the

Table 1 Clinico-pathological distribution of ESCC cases in Colombia and Chile

	Colombia (%)	Chile (%)	P
Age (yr)	63.6 ± 12.9	72.2 ± 8.9	0.003
Sex			1.000
Female	22 (47)	12 (46)	
Male	25 (53)	14 (54)	
Differentiation of tumors			0.071
Well	17 (36)	4 (15)	
Moderate	23 (49)	13 (50)	
Poor	7 (15)	9 (35)	
p16 expression ¹			0.625
Negative	21 (46)	14 (54)	
Positive ²	25 (54)	12 (46)	

¹p16 expression was not examined in 1 Colombian case because of inappropriate condition for immunostaining; ²Positive p16 expression: p16 immunostaining was observed in 10% or higher of observed carcinoma cells.

ECL direct labeling and detection kit (Amersham, UK) according to the manufacturers instructions. Amplified PCR products that appeared as a visible band after ethidium bromide staining were purified using QIAGEN PCR purification kit and directly sequenced by fluorescent dye-labeled dideoxynucleotides and cycle sequencing methods using the “Big Dye Terminator cycle sequencing kit” (PE Applied Biosystems, New Jersey, USA). In the samples where positive signal was seen only after Southern blot analysis but not in the agarose-gel electrophoresis, a second round of PCR was conducted. Sequence analysis was performed on the ABI PRISM 310 genetic analyzer (PE Applied Biosystems, New Jersey, USA). The nucleotide sequences were aligned and compared with those of known HPV types available from the GenBank database (NCBI, National Institute of health, Bethesda, MD, USA) by using BLAST 2.2 (<http://www.ncbi.nih.gov/BLAST/>).

P16^{INK4A} protein immunostaining

Sections of paraffin-embedded tissue with a thickness of 2-3 µm were placed on silane-coated glass slides, and deparaffinized by passage through xylene. After the endogenous peroxidase activity was blocked with 0.3% H₂O₂/methanol, the slides were then rehydrated with 0.01 mol/L sodium phosphate/citrate buffer, pH 8.0. For antigen retrieval, the slides were heated in 0.01 mol/L-citrate buffer, pH 6.0, at 95°C for 30 min, and left to cool for 30 min. After rinsed in 0.01 mol/L phosphate-buffered saline (PBS), pH 7.4, nonspecific antibody binding was reduced by incubating the sections with 10% fetal bovine serum in PBS for 30 min. Then, the sections were incubated overnight at 4°C with a mouse monoclonal antibody of p16 protein (1:200 dilution, GST-p16^{INK4A}, PharMingen International). After washing thoroughly with PBS, the slides were incubated with biotinylated horse anti-mouse IgG for 30 min followed by 1:100 dilution of the avidin-biotin-peroxidase complex (Vectastain elite ABC kit, Vector Laboratories, Burlingame, CA) for an additional 30 min. The peroxidase signal was visualized by treatment with DAB substrate-chromogen system (DAKO) for 8 min. Finally the sections were stained lightly with

Table 2 HPV detection in 73 cases of Colombia and Chile ESCC

	n	HPV16 (%)	P	HPV18 (%)	p
Country			0.506		0.011
Colombia	47	6 (13)		10 (21)	
Chile	26	5 (19)		0 (0)	
Sex			0.745		0.499
Female	34	6 (18)		6 (18)	
Male	39	5 (13)		4 (10)	
Age (yr)			0.316		0.212
< 60	19	1 (5)		5 (26)	
60-	22	3 (14)		2 (9)	
70-	32	7 (22)		3 (9)	
Differentiation of tumors			0.834		0.682
Well	21	4 (19)		4 (19)	
Moderate	36	5 (14)		4 (11)	
Poor	16	2 (13)		2 (13)	
p16 expression			0.190		0.515
Negative	35	3 (9)		6 (17)	
Positive ¹	37	8 (22)		4 (11)	

¹Positive p16 expression: p16 immunostaining was observed in 10% or higher of observed carcinoma cells.

hematoxylin. In statistical analysis, those having less than 10% cells stained positive were classified as negative cases and the others were regarded as positive cases^[22].

Statistical analysis

Fisher's exact test was used to examine the association between HPV status and each clinico-pathological factor including p16 expression. We used Wilcoxon rank-sum test for the comparison of age distribution. All the *P* values presented in the present study were two-sided.

RESULTS

We examined 47 Colombian cases and 26 Chilean cases. Their clinico-pathological features are summarized in Table 1. Chilean patients were older than Colombian patients, and Chilean ESCC tended to be more poorly-differentiated than Colombian tumors.

HPV was detected using GP5+/GP6+ primer pair by PCR and its presence was confirmed by Southern blot analysis using HPV-16, -18, -11 and -6 specific probes. In all HPV-negative samples, beta-globin gene was successfully amplified by PCR. Table 2 summarizes the results of HPV detection analysis. HPV-16 and -18 were detected in 11 and 10 cases, respectively. No other HPV genotypes were detected in either country. We confirmed the absence of HPV-6 and -11 by Southern blot analysis using the probes specific for HPV-6 and -11. HPV genotype was determined by sequencing analysis of L1 region fragment.

HPV-16 was detected in 6 Colombian cases (13%) and 5 Chilean cases (19%). The five positive samples from Chile were detected only after Southern blot analysis, and a second round of PCR was necessary for sequence analysis. The difference in HPV detection rate of the two countries was not statistically significant (*P* = 0.181). HPV-16 detection rate did not show any statistically significant association with sex or age. The presence of

HPV-16 genome was more frequent in well-differentiated carcinoma and p16-positive tumors but their association was not statistically significant.

HPV-18 was detected in 10 Colombia cases (21%) but not in any Chilean cases. The observed difference was statistically significant ($P = 0.011$, Fisher's exact test). HPV-18 detection rate did not show any statistically significant association with sex, age, or with p16 expression. Its detection rate was higher in well-differentiated tumors as was the case for HPV-16 but the association was not statistically significant.

DISCUSSION

In the present study, we detected HPV in 29% of ESCC specimens using PCR and Southern blot analysis. The observed frequency is similar to that obtained from the meta-analysis by Syrjänen^[4]. HPV-16 and -18 were detected in 15% and 14% of ESCC specimens, respectively. HPV-16 positive and negative tumors did not show any differences in demographic and pathological features.

The etiological role of HPV in development of ESCC is as yet unclear. There is, however, evidence that HPV plays an etiological role in development of ESCC. For example, high risk HPV E6/E7 genes have been shown to be capable of inducing immortalization in established cell lines of fetal esophageal epithelial cells and primary culture of human esophageal keratinocytes^[22,23]. HPV E6 proteins are known to bind to p53 *in vivo* and to abrogate p53-mediated repression of transcription^[24]. The mode of p53 activation at a cancer site may differ. It is suggested that the major mode of p53 inactivation may be through interaction with E6 in HPV-infected cervical tissue while both E6-p53 interaction and mutation of the p53 take place in upper aero-digestive tract carcinomas^[11].

The carcinogenic mechanism of HPV involves the binding and inactivation of pRb by HPV E7 products as well, and the expression of HPV-16 E7 protein correlates with reduced pRb levels in cervical biopsies^[25]. However, such a relationship has not been confirmed in esophageal carcinomas. On the other hand, the loss of this protein causes overexpression of p16, which is an inhibitor of cyclin-dependent kinase. This event is very frequent in cervical cancer, where HPV has been demonstrated to play an etiological role^[12]. In the present study, positive HPV-16 was more frequently detected among p16 positive cases, supporting the hypothesis that HPV may have an etiologic role in esophageal carcinogenesis. The observed association was, however, not statistically significant. On the other hand, the frequency of HPV-18 did not differ in p16-positive and -negative carcinomas. Further studies on this association seem warranted.

In the present study, the prevalence of HPV-18 was significantly lower in Chilean ESCC cases than in Colombian cases, whereas HPV-16 detection rate did not show any differences between the two countries. Regarding this observation, the prevalence of HPV in the two countries may give some insights. A study on cervical smears collected from women aged 15-69 years in Santiago, Chile^[26], showed that the most common HPV genotype is HPV-16 followed by HPV-56 and -58, while

HPV-18 is detected in 0.5 % of subjects and HPV-16 in 2.6% of subjects. In Bogota, Colombia, a study on cervical smears^[27] showed that HPV-16 prevalence is 16.1% among women aged 35 or older and HPV genotypes of 18, 39, 45, 59, and 68 are present in 13.6% of women of the same age group. Although comparison of the results obtained from these studies is difficult because of the differences in their study designs, their results suggest that HPV 18 is much less common than HPV-16 in the study areas.

The mortality of esophageal cancer is estimated to be 7.3 per 100 000 men and 3.5 per 100 000 women and the fifth leading cause of cancer death in men and the seventh in women^[28]. Interestingly, its frequency evidently varies worldwide^[29]. The high-risk areas include the so-called Asian esophageal cancer belt from eastern Turkey, Iraq, Iran, and western and northern China^[30]. The wide geographic variations in the risk of esophageal cancer suggest that environmental and genetic factors are involved in development of esophageal carcinomas, and HPV is suspected of a potential factor contributing to the high incidence of ESCC in areas of Iran, China and other countries^[31,32]. Latin America is not known to be a region where esophageal cancer risk is high. In Cali, Colombia, the local cancer registry office reported that esophageal cancer incidence (crude rate) is 6.5 per 100 000 men and 2.0 per 100 000 women, respectively, in 1995-1998^[33]. In Chile, cancer registry data are not available. However, cancer mortality, which is assessable even if cancer registry is not present, is expected to be not much different from cancer incidence because its prognosis is poor as is the case in other countries^[34]. In this country, esophageal cancer is the fifth leading cause of death from neoplastic diseases in men (5.8/100 000) and the twelfth in women (3.5/100 000)^[35]. The Hospital San Camilo, which participated in the present study, is the major medical institution in San Felipe city, Aconcagua Province. In this area, esophageal cancer mortality rate is estimated to be 6.5/100 000 persons, not evidently different from the rate in the entire nation. Thus, we can conclude that the ESCC risk is not much different in the two countries. Since Chilean ESCC cases had a higher prevalence of HPV-16 (without statistical significance), but a significantly lower prevalence of HPV-18 than in Colombian cases in the present study. The frequency of HPV-related ESCC may not be related to factors that play an important role in the incidence of ESCC in the two countries. However, the small number of cases examined makes it difficult to draw any definitive conclusion.

In conclusion, HPV-16 and -18 genotypes can be found in ESCC patients from Colombia and Chile. Further studies on the relationship between HPV-16 and -18 presence and p16 expression are needed for understanding the mechanism underlying the presence of HPV in ESCC.

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Expression of $\beta 2$ -integrin on leukocytes in liver cirrhosis

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Abstract

AIM: To analyze $\beta 2$ -integrin expression on blood leukocytes in liver cirrhosis.

METHODS: In 40 patients with liver cirrhosis and 20 healthy individuals, the evaluation of expression of CD11a (LFA-1 α), CD11b (Mac-1 α), CD11c (αX) and CD49d (VLA-4 α) on peripheral blood leukocytes was performed using flow cytometry. The analysis was carried out in groups of patients divided into B and C according to Child-Pugh's classification.

RESULTS: An increased CD11a, CD11b, CD11c and CD49d integrin expression was observed on peripheral blood leukocytes in liver cirrhosis. The integrin levels were elevated as the advancement of liver failure progressed. The highest expression of integrins occurred predominantly on monocytes. A slight expression of VLA-4 was found on lymphocytes and granulocytes and it increased together with liver failure. A positive correlation was noted between median intensity of fluorescence (MIF) expression on polymorphonuclear cells of CD11a and CD11c and CD49d ($r = 0.42$, $P < 0.01$; $r = 0.53$, $P < 0.01$, respectively) in liver cirrhosis stage C. However, no correlation was observed between integrin expression on leukocytes. The concentrations of sICAM-1, sVCAM-1, and TNF α , were significantly elevated in liver cirrhosis.

CONCLUSION: $\beta 2$ -integrin expression on leukocytes increases in liver cirrhosis decompensated as the stage of liver failure increases, which is a result of permanent activation of leukocytes circulating through the inflamed liver environment. $\beta 2$ -integrin expression on circulating leukocytes can intensify liver cirrhosis.

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Key words: $\beta 2$ -integrin; Liver cirrhosis; Flow cytometry; Leukocytes

INTRODUCTION

Integrins play a role in organ and tissue damage, by the inflammatory and immunological processes, and in autoimmune diseases^[1-4]. They participate in the regulation of leukocytes in the procoagulant activity and adhesion to infectious factors^[5]. Many integrins mediate transduction of signals that stimulate T cells^[6]. $\beta 2$ integrins participate in the presentation of antigens by antigen-presenting cells (APCs). They mediate the adhesion of T cells to APCs through the formation of immunological synapses with the help of $\beta 2$ /ICAM-1 complexes, known as the supra-molecular activation complex (SMAC). Integrin expression strongly depends on cellular energy metabolism. Activated leukocytes express $\beta 1$ and $\beta 2$ that integrins play a key role in leukocytes mobilization to circulate to inflammatory sites^[7]. Monocytes and granulocytes are important in organic defense against bacterial and viral infections. LFA-1 and Mac-1 are involved in other functions of neutrophils, such as phagocytosis, degranulation, and apoptosis^[2].

Inflammatory and immunological reactions in the liver are accompanied by peripheral blood leukocytes infiltration in the tissue^[8]. Leukocyte recruitment takes its course with the participation of adhesive molecules presented by capillary endothelial cells^[8,9]. The particular stages of recruitment, such as leukocyte rolling, adhesion, and transmigration, depend on signals released by endothelial cells. Activated leukocytes expressed adhesion molecules $\beta 2$ -integrins CD11a (LFA-1 α), CD11b (Mac-1 α), CD11c (αX), and $\beta 1$ -integrins CD49d (VLA-4 α) on their surfaces^[1,3,4]. Integrins mediate in interactions between cells and extracellular matrix (ECM) proteins. ICAM-1 (ligand for LFA-1 α and Mac-1 α), ICAM-2 (ligand for LFA-1 α), VCAM-1 (ligand for VLA-4 α), extracellular matrix proteins, such as collagen, laminine, vitronectine as well as blood clotting proteins (fibrinogen, factor X, kininogen) are ligands for integrin receptors on leukocytes. Products released by microorganisms, that can facilitate penetration into cells, can be ligands for integrins^[5]. Cytokines released in inflammatory sites induce the expression of endothelial ligands that activate circulating leukocytes^[10].

Inflammation in liver cirrhotic tissue and in the systemic circulation affects peripheral leukocytes $\beta 2$ -integrin receptors. In this study, the evaluation of $\beta 2$ -integrin expression

on monocytes, lymphocytes, and granulocytes of peripheral blood in liver cirrhosis was carried out. The influence of liver failure stage on the expression of $\beta 2$ -integrin leukocyte receptors was taken into account.

MATERIAL AND METHODS

Forty patients (28 men and 12 women; age 50 ± 13 years) with post-alcoholic liver cirrhosis were enrolled in this study. Liver biopsy of the right lobe established the liver cirrhosis. Clinical characteristics of patients are given in Table 1. According to Child-Pugh's classification^[11], patients were divided into 2 groups: B ($n = 21$) and C ($n = 19$). Those overusing alcohol with immunosuppression therapy, hepatotropic viral infections (HBV, HCV, CMV) or fever were excluded from the study. The control group consisted of 20 healthy individuals (12 women and 8 men; age 40 ± 7 years).

Leukocyte population

Peripheral blood leukocyte population (monocytes, lymphocytes, granulocytes) was determined by flow cytometry (Coulter, USA). Leukocytes were identified by their forward and orthogonal light scatter characteristics on immunological gate. The percentage of leukocyte population and median intensity of fluorescence (MIF) of integrin receptors were determined using mononuclear antibodies CD11a (CD11a PE, DAKO Cytomation, Denmark), CD11b (CD11b PE, DAKO Cytomation, Denmark), CD11c (CD11c PE, DAKO Cytomation, Denmark), CD49D (CD11a PE, DAKO Cytomation, Denmark), and CD14 (CD14 FITC, DAKO Cytomation, Denmark). Two millilitres of blood was collected in plastic tubes containing sodium citrate. Five microlitres of mAb was added to 50 μ L of blood and incubated for 15 min at room temperature in the dark. Erythrocytes were eliminated by adding diluting-lysing fluid (ImmunoPrep-Reagenziensystem ABC, Coulter). A minimum of 10 000 leukocytes was analysed in each sample.

Tumor necrosis factor alpha (TNF α , R&D, England), sICAM-1 (sICAM, R&D, England), sVCAM-1 (sVCAM-1, R&D, England) concentrations were determined in the blood sera using ELISA methodology. Leukocyte counts was carried out in hematological analyzer Sysmex K-1000 (Japan). Ethical approval for research was obtained from the Local Ethics Committee in the Medical University.

Statistical analysis

The results were expressed as median with range values and mean \pm SD. Statistical analysis was performed by non-parametrical Mann-Whitney *U*-test. Result correlation was calculated with the use of Spearman test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Monocytes

The expression of CD11a (LFA-1a) receptors on peripheral blood monocytes was increased in liver cirrhosis

Table 1 Clinical characteristics of patients with liver cirrhosis

Characteristic	Child-pugh B	Child-pugh C
Encephalopathy (<i>n</i>)	2	10
Ascites (<i>n</i>)	9	19
Median bilirubin (range) (mg/L)	34 (9–78)	69 (22–139)
Median albumin (range) (g/L)	29 (22–33)	22 (19–29)
Median prothrombin time (range) (s)	19 (17.2–23.3)	23 (18.2–26.8)

Table 2 $\beta 2$ -integrin expression on peripheral blood leukocytes and sICAM-1 and TNF- α serum concentrations in liver cirrhosis (stages B and C) and in healthy subjects

Leukocytes	Receptors	Healthy subjects	Liver cirrhosis	
		(<i>n</i> = 20)	Stage B (<i>n</i> = 21)	Stage C (<i>n</i> = 19)
Monocytes	CD14 (%)	26.2 \pm 2.3	26.1 \pm 6.3	26.8 \pm 3.9
	MIF ¹	2.9 \pm 1.5	8.7 \pm 6.9	8.1 \pm 6.1
	CD11a (%)	99.9 \pm 0.09	99.9 \pm 0.08	99.9 \pm 0.8
	MIF ¹	14.8 \pm 2.4	15.1 \pm 1.8 ^a	16.3 \pm 1.7 ^b
	CD11b (%)	99.8 \pm 0.08	99.5 \pm 0.7	99.8 \pm 0.2
	MIF ¹	18.4 \pm 4.6	18.7 \pm 4.8 ^a	22.2 \pm 4.4 ^a
	CD11c (%)	99.5 \pm 0.5	97.5 \pm 4.5	96.6 \pm 7.0
	MIF ¹	6.5 \pm 1.3	7.0 \pm 1.5	7.4 \pm 2.1
Granulocytes	CD49d (%)	63.8 \pm 14.7	66.9 \pm 22.5	66.2 \pm 21.2
	MIF ¹	1.39 \pm 0.43	1.85 \pm 0.72	1.52 \pm 0.68
	CD11a (%)	99.3 \pm 0.5	98.0 \pm 4.8	94.7 \pm 0.5
	MIF ¹	4.4 \pm 0.4	4.3 \pm 0.5	4.4 \pm 0.7
	CD11b (%)	99.5 \pm 1.1	99.5 \pm 0.7	98.6 \pm 2.7
	MIF ¹	10.5 \pm 2.4	10.0 \pm 2.8 ^a	12.1 \pm 2.6
	CD11c (%)	87.0 \pm 5.0	80.8 \pm 11.4	71.9 \pm 12.4
	MIF ¹	2.8 \pm 0.4	2.7 \pm 0.7	3.0 \pm 0.9
Lymphocytes	CD49d (%)	4.4 \pm 2.4	4.6 \pm 3.0	6.3 \pm 3.9
	MIF ¹	0.13 \pm 0.01	0.17 \pm 0.05 ^b	0.17 \pm 0.03 ^b
	CD11a (%)	98.1 \pm 0.8	97.2 \pm 1.7	97.6 \pm 1.3
	MIF ¹	8.0 \pm 1.2	9.7 \pm 1.6 ^b	9.9 \pm 2.0 ^b
	CD11b (%)	23.7 \pm 5.3	24.5 \pm 5.2	24.0 \pm 8.0
	MIF ¹	0.56 \pm 0.12	0.61 \pm 0.18 ^a	0.75 \pm 0.24
	CD11c (%)	25.2 \pm 6.6	23.2 \pm 5.6	29.4 \pm 7.3
	MIF ¹	0.77 \pm 0.12	0.91 \pm 0.33	1.0 \pm 0.25 ^b
sICAM-1 (μ g/L)		254 \pm 74	839 \pm 34 ^b	957 \pm 328 ^b
TNF α (μ g/L)		1.58 \pm 0.22	2.89 \pm 1.34 ^b	2.98 \pm 1.42 ^b

¹Medial intensity of fluorescence; ^a*P* < 0.05 vs Child-Pugh C stage, ^b*P* < 0.01 vs healthy subjects (Mann-Whitney *U*-test).

patients as compared with healthy subjects (Table 2). Progressive liver damage resulted in increased population and number of receptors for this integrin on monocytes. The differences in CD11a MIF values between patients in B and C stages were statistically significant (*P* < 0.05). There was a positive correlation between the expression of CD11a and CD11c receptors on monocytes in the control group and in B and C stages of liver cirrhosis (*r* = 0.73, *P* < 0.01; *r* = 0.39, *P* < 0.05; *r* = 0.56, *P* < 0.01, respectively, Table 3). Moreover, a positive correlation was found between monocyte CD11a MIF and CD11a expression on lymphocytes in patients in stage B. However, no other significant relations were observed between CD11a expression and other integrins on other leukocytes.

Table 3 The correlation of $\beta 2$ -integrin expression on leukocytes and sICAM level in blood sera from liver cirrhosis (stages B and C)

	CD11a	CD11b	CD11c	CD49d
Monocytes			Monocytes	
CD11a		$H^1: r = 0.6, P < 0.01$	$B^2: r = 0.39, P < 0.05$ $C^3: r = 0.56, P < 0.01$ $H^1: r = 0.73, P < 0.01$	NS ⁴
	NS ⁴	NS ⁴	Granulocytes	NS ⁴
	$B^2: r = 0.56, P < 0.01$	NS ⁴	Lymphocytes	NS ⁴
Granulocytes			Monocytes	
CD11a	NS ⁴	NS ⁴	$C^3: r = 0.47, P < 0.04$	NS ⁴
			Granulocyte	
			$C^3: r = 0.42, P < 0.01$ $H^1: r = 0.54, P < 0.05$	$C^3: r = 0.53, P < 0.01$
	NS ⁴	NS ⁴	Lymphocytes	
Lymphocytes			Monocytes	
CD11a	NS ⁴	NS ⁴	NS ⁴	NS ⁴
	NS ⁴	NS ⁴	Granulocytes	
			NS ⁴	NS ⁴
			Lymphocytes	
		$C^3: r = 0.51, P < 0.02$	NS ⁴	$C^3: r = 0.51, P < 0.02$

¹Healthy subjects; ²B stage of liver cirrhosis; ³C stage of liver cirrhosis; ⁴Not significant. Spearman test.

CD11c MIF increased slightly on monocytes in B and C stages of liver cirrhosis ($P > 0.05$). Furthermore, CD 49d expression in subsequent stages of liver cirrhosis was slightly elevated on monocytes ($P > 0.05$). There was no dependence observed between VLA-1a receptor expression and the serum concentration of soluble forms of ICAM-1, VCAM-1, and TNF α .

Granulocytes

The expression of CD11a on granulocytes in healthy subjects and liver cirrhosis (independently the stages B and C) was comparable. CD11b receptor expression on granulocytes in severe liver cirrhosis was significantly elevated ($P < 0.05$, controls *versus* stage B). In contrast, no significant difference was observed in the expression of CD11c on granulocytes between the liver cirrhosis and control groups. CD49d expression on granulocytes was approximately 10 times lower than that on monocytes, although it increased together with the stage of liver damage ($P < 0.01$ *versus* the controls). A positive correlation was observed between CD11a and CD11c MIF on granulocytes ($r = 0.42, P < 0.01$) in liver cirrhosis stage C. Also, a positive correlation between CD11a expression on granulocytes and CD49d in liver disease stage C ($r = 0.53, P < 0.01$) was found.

Lymphocytes

LFA-1, among other $\beta 2$ -integrins, had the highest expression on lymphocytes. In liver cirrhosis, higher values of LFA-1 on leukocytes were observed, and were found to be increased with the advancement of liver insufficiency (stage B: 9.7 ± 1.6 , stage C: 9.9 ± 2.0 , healthy: 8.0 ± 1.2 ; $P < 0.05$ *vs* controls). A significantly lower expression of Mac-1, αX , and VLA-4 was noted on lymphocytes, while increased expression was detected in liver cirrhosis. The elevation of CD11b and CD11c expression was found to be affected by

the stage of liver failure, whereas VLA-4 expression was not influenced by the condition. A positive correlation was noticed between LFA-1 expression and Mac-1 expression (stage C: $r = 0.51, P < 0.02$), as well as VLA-4 expression (stage C, $r = 0.51, P < 0.02$).

We did not observe any correlation between the concentrations of soluble forms of ICAM-1 and VCAM-1 and the expression of integrins in particular stages of liver cirrhosis and in the controls. Stage B of liver cirrhosis was the only stage, in which a negative correlation was observed between sICAM-1 and CD11a expression on lymphocytes ($r = 0.49, P < 0.03$). On contrary, a positive correlation was noted between TNF- α concentration and sICAM-1 level in liver cirrhosis stage B ($r = 0.68, P < 0.01$).

DISCUSSION

Studies showed that the stage of liver failure has a significant impact on $\beta 2$ -integrins by the intensification of their expression on leukocytes. Liver cirrhosis was associated with elevated expression of $\beta 2$ -integrins CD11a, CD11b, CD 11c, and CD49d on leukocytes surfaces. The results indicated leukocyte stimulation in liver cirrhosis, and that a cell population of higher function can potentially appear. It was observed that the expression of $\beta 2$ -integrins on monocytes, granulocytes, and lymphocytes increased in patients with liver cirrhosis. The only exception was LFA-1 on granulocytes, whose expression did not differ as compared to the controls. Monocytes had the highest $\beta 2$ -integrin expression on the surface, indicating that monocytes have an important role in the pathogenesis of liver cirrhosis. The population of monocytes with $\beta 2$ -integrin expression increases with the stage of liver failure. It causes the elevation of their activity in relation between cells and ECM proteins. Circulating monocytes

are precursors of dendritic cells and macrophages of the liver. Activated by a liver inflammatory environment, they expose on their surfaces adhesion molecules that enable active participation in the intensification of inflammatory and immunological processes in the liver. Immunological disorders belong to the main phenomena in the pathogenesis of liver cirrhosis. Moreover, it has been shown that liver cirrhosis is accompanied by high concentrations of TNF- α and soluble adhesive molecules sICAM-1 and sVCAM-1, whose concentrations did not correlate with β 2-integrin expression on leukocytes in our studies.

The activation of endothelial cells has a crucial role in modulation of leukocyte functions in liver cirrhosis. Endothelial soluble adhesive molecules, induced by pro-inflammatory cytokines, are the markers of the functional condition of endothelial cells. The role of adhesive molecules in the pathogenesis of liver cirrhosis and portal hypertension has not been fully explained yet. Integrins participate in adhesion and migration of leukocytes in the microcirculation of highly perfused organs (the liver, lungs)^[12]. Endothelial cell incubation with antibodies against integrin receptors inhibits monocyte adhesion^[7]. The administration of anti-LFA-1 or anti-ICAM-1 antibodies diminishes significantly the stage of hepatocyte damage. It is suggested that T lymphocytes, infiltrating the liver through LFA-1/ICAM-1 interactions, cause the damage of hepatocytes by releasing toxic substances and oxygen radicals^[6]. Damaged endothelial cells present adhesive molecules (selectins, ICAM-1, VCAM-1) on their surfaces and influence flowing leukocytes. Activation signals are transmitted directly cell to cell or by the contact of cells with soluble adhesive molecules. Inflammatory and immunological phenomena have common paths, that are frequently paralleled, and leukocytes are main effector cells.

Integrin expression on leukocytes undergoes regulation by pro-inflammatory cytokines such as TNF- α and IL-8. CD11b expression on leukocytes correlates with IL-6 level and the stage of liver failure^[13]. Increased LFA-1, LFA-3, and ICAM-1 expression on leukocytes and TNF- α production in post-alcoholic liver cirrhosis has been observed^[14]. Peripheral blood monocytes showed activity and elevated expression of TNF- α which correlated with liver disease activity (Child-Pugh stage B and C)^[15]. The concentrations of TNF- α and soluble adhesive molecules are significantly higher in the advanced phase of cirrhosis ($P < 0.05$, Child-Pugh class B and C *versus* A) and can reflect hemodynamic alterations in the liver^[16]. This is accompanied by the activation of monocytes and increased intracytoplasmatic expression of TNF- α ^[17]. Bacterial infections in the alimentary tract play an important role in cellular immunological disorders in patients with liver cirrhosis. A correlation between TNF- α level in blood serum and TNF- α contained in monocytes occurs in decompensated liver cirrhosis with a high concentration of LPS in blood. Antibacterial treatment diminishes monocyte activity and TNF- α -production activity^[17]. Inflammatory mediators, cytokines (TNF- α), and selectins elevate CD11b/CD18 expression on granulocytes. CD11b/CD18 cell population is increased in inflamed tissues. Accumulation of leukocytes with LFA-1, Mac-1,

and VLA-4 expression occurs in liver parenchyma near metastasis focuses^[18].

Patients with liver cirrhosis reveal the immunological system disorders and immunodeficiency, and the activation of certain immunological responses. Luna-Casad *et al*^[19] showed an increase in ICAM-1, LFA-3, and Mac-1 expression on lymphocytes and LFA-3 on monocytes. This correlated with the severity of liver cirrhosis and immunological disorders.

Wong *et al*^[12] showed that most stimulated leukocytes (approximately 80%) adhere to sinusoids and the rest (20%) to post-sinusoidal venules. The adhesion of stimulated leukocytes is independent of P, E, and L-selectin presence in the liver microcirculation. The adhesion of neutrophils to ICAM-1 is possible due to molecules exposed on CD11a and CD11b surfaces^[3]. Bacterial infections are accompanied by activation of peripheral blood leukocytes and their recruitment in the liver. LFA-1 immunoneutralization reduced leukocyte adhesion to a great extent (by 55%), decreased aminotransferase activity by 65%, and reduced apoptosis in the liver by 45%^[20]. It has been shown that LFA-1 plays an important role in the development of liver deficiency through leukocytes. LFA-1 expression in inflammatory infiltrates is elevated in PBC, which points to the role of activated lymphocytes in bile duct damage^[21]. Hepatotropic virus infections (e.g. HHV-6) also induce LFA-1 and VLA-1 expression on lymphocytes infiltrating the liver^[22].

Liver resident macrophages—Kupffer's cells constitutively show high LFA-1, Mac-1, and ICAM-1 expression^[23]. The studies performed on transgenic mice (CD11b-DTR) revealed that activated macrophages participate both in tissue damage and repair in inflammatory processes. Macrophages are capable of diminishing liver fibrosis through ECM protein degradation^[24].

In summary, β 2-integrins play an important role in the development of liver cirrhosis. The stage of disease advancement activates peripheral blood leukocytes, which results in the intensification of liver inflammatory and immunological processes. The activation of leukocytes is a complex phenomenon, influenced by endo- and exogenic factors. The blockade of integrin expression on leukocytes may constitute an important therapeutic aspect of liver cirrhosis.

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RAPID COMMUNICATION

Expression of p53, Bax and Bcl-2 proteins in hepatocytes in non-alcoholic fatty liver disease

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Abstract

AIM: To analyze the protein expression essential for apoptosis in liver steatosis.

METHODS: The expression of proapoptotic proteins p53, Bax, and antiapoptotic Bcl-2 in hepatocytes with steatosis (SH) and without steatosis (NSH) was evaluated in 84 patients at various stages of non-alcoholic fatty liver disease (NAFLD).

RESULTS: Immunohistochemical staining of liver tissue showed the activation of p53 protein in SH and NSH with increased liver steatosis, diminished Bcl-2 and slightly decreased Bax protein. Positive correlation was found between the stage of liver steatosis with p53 expression in SH ($r = 0.54$, $P < 0.01$) and NSH ($r = 0.49$, $P < 0.01$). The antiapoptotic protein Bcl-2 was diminished together with the advancement of liver steatosis, especially in non-steatosed hepatocytes ($r = 0.43$, $P < 0.01$).

CONCLUSION: Apoptosis is one of the most important mechanisms leading to hepatocyte elimination in NAFLD. The intensification of inflammation in NAFLD induces proapoptotic protein p53 with the inhibition of antiapoptotic Bcl-2.

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Key words: Apoptosis; Non-alcoholic liver disease; p53; Bcl-2; Bax; Immunohistochemistry

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INTRODUCTION

Liver steatosis results from triglyceride accumulation in hepatocytes in the course of many diseases^[1]. The failure of energetic processes in hepatocytes leads to progressive lipid increase in cytoplasm^[2]. The most common cause of liver steatosis is alcohol abuse (alcoholic liver fatty disease-ALFD)^[3]. The disease accompanies hyperalimentation, obesity, metabolic syndromes, and hyperlipidemia^[4,5]. The phenomenon can be the result of side effects of some contraceptive, NSAID, antimetabolic and other drugs^[1,3]. Non-alcoholic fatty liver disease (NAFLD) is diagnosed by excluding alcohol abuse and other liver diseases and confirmed by histological changes in the liver and increased aminotransferase activity.

A variety of pathological conditions associated with NAFLD could explain the pathogenesis of the disorder. There is a hypothesis concerning multifactorial conditioning of the disease. Different mechanisms are assumed to lead to NAFLD, including imbalanced fatty acid supply, hyperglycemia, upset hormonal balance between hormones responsible for anabolic and catabolic activities in the portal circulation, and endotoxemia in starvation^[2,3]. Oxidative stress that damages lipid peroxidation and mitochondria occurs in chronic liver injury (metabolic disturbances, iron deposition, high fatty acid concentrations). Fatty acid cytotoxic activity is suggested to influence cell survival. Long term accumulation of lipids may lead to hepatocyte necrosis or apoptosis^[6]. The mechanisms underlying cell death have not been explained yet. The significant influence of inflammatory and immunological factors, cytokines, and chemokines is probable^[7]. The stimulation of mechanisms underlying apoptosis, with the contribution of p53 in hepatocytes has been observed in mice in laboratory conditions^[6,8,9].

The aim of our study was to evaluate the expression of proapoptotic p53 and Bax and antiapoptotic Bcl-2 in hepatocytes of patients with liver steatosis. The expression of these proteins, depending on disease advancement and enhancement of steatosis was analyzed in the study.

MATERIALS AND METHODS

Patients

Examinations were performed in 84 patients with liver steatosis (35 women and 49 men, median age 43 years, range 27-62 years). Steatosis was due to obesity, glucose intolerance and chronic use of hepatotoxic drugs. Patients

infected with hepatitis B virus (HBV) and hepatitis C virus (HCV) were excluded from the study. Liver steatosis was confirmed by ultrasonography and biopsy. Liver dysfunction was confirmed by increased aminotransferase values that persisted for longer than 6 mo. The Brunt classification was used to perform the histological analysis of steatosis advancement^[10].

Methods

Right lobule liver oligobiopsies were conducted in all patients. Liver biopsies were performed using the Hepafix System 1.6 (Braun, Melsungen, Germany) before treatment. Liver tissues obtained from biopsies were placed in 4% formaldehyde buffered solution for 24 h, then fixed and paraffin-embedded. Five- μ m thick sections were routinely stained with hematoxylin and eosin as well as picric acid.

Bcl-2: Tissue sections were put into FSG 120 (FSG120-T/T Controlled Antigen Retrieval, Milestone, Italy) at 110°C after paraffin was removed and hydration performed in citric buffer (pH 6.0). The pressure was adjusted to 2 bar for 10 min to obtain antigen retrieval. After cooling, the samples were placed in 3% hydrogen peroxide solution for 5 min, then washed in distilled water and placed in fresh buffered Tris for 5 min. The histological preparation was covered with the primary antibody Bcl-2 at 1:40 dilution (anti-human Bcl-2 coprotein, DakoCytomation, Denmark), incubated at room temperature for 30 min and placed in a fresh Tris bath for 5 min. Then, the secondary antibody was added (biotinylated link universal from the commercial kit LSAB: DakoCytomation, Denmark), incubated at room temperature for 30 min before washing in fresh Tris for 5 min. Another secondary antibody was added (streptavidin-HRP from LSAB kit) for 30 min and the section was placed in fresh Tris for 5 min. The preparation was covered with DAB stain (3, 3-diaminobenzidine tetrahydrochloride, DAB Chromogen, DakoCytomation, Denmark) for 10 min and then bathed in distilled water.

p53: The samples were deprived of paraffin, hydrated and placed in a microwave at 600V for 20 min. The procedures described above using anti p53 antibodies in dilution of 1:50 (anti-human p53 protein, DakoCytomation, Denmark) were repeated.

Bax: Preparations were prepared as above. Bax protein was detected using the commercial kit CSA (CSA, DakoCytomation, Denmark) according to the manufacturer's recommendations. The primary Bax antibody was used at dilution 1:1000 (anti-human Bax protein, Dako, Denmark). Then, the preparation was processed.

Using paraffin at a high melting point (65°C) and antigen retrieval in the microwave, we could not detect Bcl-2 and Bax proteins in hepatocytes. On the other hand, using low-melting point paraffin (55°C) and a FSG 120 machine for routine pressurized high temperature antigen retrieval, positive immunohistochemical reactions for Bcl-2 and Bax in hepatocytes were obtained.

The positive reaction showed as brown color of the cytoplasm for Bcl-2 and Bax, and the nuclei for p53. We calculated hepatocyte percentage (the amount of cells with positive reaction in ratio to 100 cells) containing those

Table 1 Clinical characteristics of patients with liver steatosis

Clinical characteristic	Grade of steatosis (% of steatosis hepatocytes)		
	< 33% (group I)	33%-66% (group II)	> 66% (group III)
<i>n</i>	24	35	25
BMI	28 kg/m ²	30 kg/m ²	32 kg/m ²
Cholesterol (mmol/L)	5.50 \pm 0.75	5.99 \pm 1.29	6.80 \pm 2.30
Triglyceride (mmol/L)	2.60 \pm 1.27	2.78 \pm 1.44	3.16 \pm 1.11
Bilirubina (μ mol/L)	11.97 \pm 5.13 ^{b,d}	17.1 \pm 6.84	27.36 \pm 20.52
ALT (μ kat/L)	0.84 \pm 0.35 ^{b,d}	1.70 \pm 1.12	1.67 \pm 0.38
AST (μ kat/L)	0.60 \pm 0.33 ^{b,d}	1.19 \pm 0.50	1.87 \pm 1.80
ALP (μ kat/L)	1.12 \pm 0.27 ^{b,d}	1.72 \pm 0.45	2.10 \pm 0.92
GGT (μ kat/L)	0.75 \pm 0.33 ^{b,d}	2.61 \pm 3.42 ^d	5.06 \pm 4.34
Total protein (g/L)	7.4 \pm 0.4 ^d	7.0 \pm 0.5 ^d	6.4 \pm 0.5
Albumin (g/L)	4.5 \pm 1.2 ^d	4.0 \pm 0.2 ^d	3.4 \pm 0.7

^b*P* < 0.01 vs group II, ^d*P* < 0.01 vs group III (non-parametrical Student's-*t* test).

proteins in 5 fields of vision (\times 400) of one slide under a light microscope. Expression of Bcl-2, p53 and Bax was analyzed in steatotic (micro- and macro-droplet steatosis) and non-steatotic hepatocytes. Ethical approval for research was obtained from the Ethics Committee of Medical University.

Statistical analysis

The results were presented as the mean cell number (p53, Bcl-2 or Bax positive hepatocytes) \pm SD. The statistical analysis was carried out using non-parametrical Student's-*t* test and Spearman's correlation test.

RESULTS

Histological analysis of liver biopsies showed mixed micro- and macro-droplet steatosis of hepatocytes occurring mainly in the 2nd and 3rd zones of the lobules. Inflammation and fibrosis were not present in group I (hepatocyte steatosis < 33%), but were present in group II. Moderate periportal and interstitial fibrosis, small necrotic focuses of hepatocytes without necrosis and single lymphocyte clusters were noted in group III (steatohepatitis). Malformation in portal spaces was not observed. Table 1 presents the characteristics of the examined groups. The activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyltransferase (γ -GT) were increased, total protein and albumin concentrations decreased significantly together with the stage of liver steatosis. There was no significant relationship between the activity of aminotransferase (ALT, AST) and the expression of p53, Bax, Bcl-2 in hepatocytes with SH and HNS.

Bax

Bax expression was significantly higher in steatosed hepatocytes with SH (68% \pm 23%) than in non-steatosed hepatocytes (NSH) (59% \pm 21%, *P* < 0.03) in the patients with NAFLD (Table 2). The highest Bax expression was observed in SH in group I with slight liver steatosis. The intensification of inflammation and fibrosis led to an

Table 2 Percentage of hepatocytes containing pro- and antiapoptotic proteins depending on the stage of liver steatosis (mean \pm SD, %)

	Total	Group I	Group II	Group III
Bax NSH ¹	59 \pm 21	66 \pm 15	52 \pm 27	57 \pm 19
Bax SH ²	68 \pm 23	71 \pm 21	63 \pm 28	67 \pm 18
p53 NSH ¹	24 \pm 12	18 \pm 14 ^d	21 \pm 9 ^d	35 \pm 9
p53 SH ²	22 \pm 17	14 \pm 18 ^{b,d}	20 \pm 13 ^d	33 \pm 14
Bcl2 NSH ¹	55 \pm 26	77 \pm 21 ^{b,d}	46 \pm 20	49 \pm 27
Bcl2 SH ²	44 \pm 27	52 \pm 37	42 \pm 20	41 \pm 24

¹Non-steatosed hepatocytes; ²Steatosed hepatocytes. ^b $P < 0.01$ vs group II, ^d $P < 0.01$ vs group III (non-parametrical Student's-*t* test).

initially insignificant decrease in Bax expression (mainly in NSH). The group of patients with steatohepatitis showed an increased Bax expression mainly in SH. We did not observe any relation between the stage of liver steatosis and the expression of Bax in hepatocytes (Table 3). The expression of p53 in SH ($r = 0.32$, $P < 0.01$) and NSH ($r = 0.33$, $P < 0.01$) was elevated together with the increase in Bax expression. The relationship between Bax expression in NSH and p53 expression in SH and NSH was weak. Together with the increase in alkaline phosphatase in steatohepatitis, Bax expression was enhanced in SH and NSH ($r = 0.57$, $P < 0.01$ and $r = 0.59$, $P < 0.01$ respectively). Gamma-glutamyltransferase elevation was also accompanied by the increase in Bax expression in SH ($r = 0.38$, $P < 0.01$) and NSH ($r = 0.33$, $P < 0.01$).

p53

The expression of p53 was approximately 10% lower in steatosed hepatocytes than in hepatocytes without steatosis (difference was not statistically significant) (Table 2). In the patients with liver steatosis without inflammation (group I), p53 expression in SH and NSH was significantly lower than in the patients with steatohepatitis (group III). There was a positive correlation between p53 expression and the stage of liver steatosis ($r = 0.49$, $P < 0.01$ in NSH; $r = 0.54$, $P < 0.01$ in SH) (Table 3). The level of albumin was negatively correlated with p53 expression in SH ($r = -0.44$, $P < 0.01$) and NSH ($r = -0.69$, $P < 0.01$). The concentrations of ALP and GGT were positively correlated with positive p53 in SH ($r = 0.36$, $P < 0.01$ and $r = 0.32$, $P < 0.01$, respectively) and NSH ($r = 0.25$, $P < 0.03$; $r = 0.34$, $P < 0.01$, respectively).

Bcl-2

Bcl-2 expression diminished in steatosed and non-steatosed hepatocytes in accordance with the stage of liver steatosis. As far as liver steatosis was concerned, there was a statistically significant difference between positive SH, NSH and Bcl-2 (44% \pm 27%, 55% \pm 26% respectively, $P < 0.05$) (Table 2). Group I had the highest expression of Bcl-2 in NSH and a lower expression in SH (77% \pm 21%, 52% \pm 35%, respectively). On the other hand, advanced stage of liver steatosis had a statistically significant decrease in Bcl-2 expression in SH and NSH. A negative correlation was observed between Bcl-2 in NSH and the stage of liver

Table 3 Correlation of biochemical degree of liver damage and expression of pro- and anti-apoptotic proteins in hepatocytes with the stage of liver steatosis

	<i>r</i>
ALT	0.6 ^b
AST	0.37 ^b
ALP	0.55 ^b
GGT	0.53 ^b
Total protein	-0.54 ^b
Albumines	-0.34 ^b
Bax NSH ¹	NS ³
Bax SH ²	NS ³
p53 NSH ¹	0.49 ^b
p53 SH ²	0.54 ^b
Bcl2 NSH ¹	-0.43 ^b
Bcl2 SH ²	NS ³

¹Non-steatosed hepatocytes; ²Steatosed hepatocytes; ³Non-statistical correlation. ^b $P < 0.01$ vs the stage of liver steatosis (non-parametrical Spearman test).

steatosis ($r = -0.43$, $P < 0.01$) (Table 3). However, such a relationship was not observed between the expression of Bcl-2 in NSH and the stage of the disease. The elevation of Bcl-2 expression in NSH increased Bcl-2 expression in SH ($r = 0.46$, $P < 0.01$), Bax expression in NSH ($r = 0.53$, $P < 0.01$) and SH ($r = 0.57$, $P < 0.01$). We did not observe any relationship between the percentage of positive Bcl-2 in NSH and the expression of p53 in SH and NSH. However, there was a correlation between Bax-2 expression in SH and p53 expression in SH ($r = 0.42$, $P < 0.01$) and NSH ($r = 0.36$, $P < 0.01$). No correlation was observed between Bcl-2 expression, alkaline phosphatase and γ -GT activities.

DISCUSSION

The study revealed that the stage of liver steatosis increased the expression of proapoptotic proteins, mainly p53, in hepatocytes with and without steatosis. Antagonistic protein Bcl-2 was diminished together with the advancement of liver steatosis, especially in normal hepatocytes. Proapoptotic protein Bax seemed to play a minor role in the process of steatosis. Its expression was slightly decreased when steatosis was intensified. There was an apparent difference between hepatocytes with steatosis containing more Bax protein and hepatocytes without steatosis. The pathogenesis of NAFLD is not entirely clear. Triglycerides, localized in the cytoplasm of hepatocytes, are the main component of lipids. Accumulation of lipids can result from insulin resistance, damaged disposal of triglycerides from the cells, β -oxidation damage in mitochondria, or very low density lipoproteins^[11]. It has been suggested that free fatty acids (FFA) can play a crucial role in steatosis intensification and necrotic-inflammatory processes. Damage of lysosome integrity, which results in cathepsin B and TNF- α release, is due to the effect of lipotoxicity. The degree of lysosome instability correlates with NAFLD activity^[12].

Cellular free fatty acids show cytotoxic effects, such as

elevation of cytochrome P450 activity^[13]. Steatosed hepatocytes are more sensitive to endotoxin, which can result from intracellular IL-10 mRNA reduction and IFN- γ mRNA concentration elevation^[13]. It is assumed that chronic oxidative stress in NAFLD influences steatosis progression^[14]. Oxidative stress is generated by free fatty acids, pro-inflammatory cytokines, TNF alpha is activated by cytochrome P450 2E1. The disturbances of lipid peroxidation in hepatocytes, which can lead to their extensive accumulation, is the consequence of oxidative stress. Seki *et al*^[15] stated that intracellular increase in markers of lipid peroxidase and oxidative DNA damage correlate with the stage of necroinflammatory changes of steatosis in patients with NASH. Chronic oxidative stress induces overexpression of cytochrome P450 2E1 (CYP2E1) in hepatocytes, causing their damage. The mechanism underlying cell death with the participation of CYP2E1 has not been explained yet. Chronic overexpression of p4502E1 increases epidermal growth factor/c-Raf signaling in hepatocytes. Studies conducted by Schattenberga *et al*^[16] have proved that hepatocytes exposed to chronic oxidative stress have different sensitivity to noxious stimuli. Cellular resistance to proapoptotic factors has observed in hepatocytes with overexpression of p4502E1 after sensitized by polyunsaturated fatty acids.

Incubation of pancreatic beta-cells with FFA results in a significant increase in cell death due to apoptosis^[17]. The cells have a significant drop in Bcl-2 mRNA expression with no change in Bax mRNA expression^[17]. Cell death induced by FFA can be blocked by caspase, serine protease, and ceramide synthesis inhibition, suggesting that FFA is the cause of pancreatic beta-cell reduction and leads to peripheral insulin resistance, which plays a role in obesity and liver steatosis^[18]. It was observed that FFA administration results in proapoptotic p53 increase and anti-proliferative effect in Caco-2 cells. There is also a simultaneous drop in the level of apoptosis suppressor protein Bcl-2 expression^[19].

TNF alpha and CYP2E1, which are more significantly expressed in patients with chronic hepatitis C and steatosis than in those without steatosis, have a role in liver steatosis progression^[20]. Ji *et al*^[21] suggested that incubation of HepG2 cells with palmitic acid could induce cell death via mitochondria-mediated apoptosis. They also stated that Bax expression is significantly increased but Bcl-2 expression is moderately decreased^[21], indicating that liver steatosis influences expression of pro- and anti-apoptotic proteins. Apoptosis is one of the most important mechanisms underlying hepatocyte elimination in non-alcoholic steatohepatitis. The intensification of inflammatory changes in NAFLD induces proapoptotic protein (p53) with the inhibition of antiapoptotic protein (Bcl-2). However, the explanation of mutual relationships between apoptosis and liver steatosis needs further investigation.

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Aggressive treatment of acute anal fissure with 0.5% nifedipine ointment prevents its evolution to chronicity

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a significant healing rate for acute anal fissure and might prevent its evolution to chronicity.

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Abstract

AIM: To investigate the efficacy of topical application of 0.5% nifedipine ointment in healing acute anal fissure and preventing its progress to chronicity.

METHODS: Thirty-one patients (10 males, 21 females) with acute anal fissure from September 1999 to January 2005 were treated topically with 0.5% nifedipine ointment (t.i.d.) for 8 wk. The patients were encouraged to follow a high-fiber diet and assessed at 2, 4 and 8 wk post-treatment. The healing of fissure and any side effects were recorded. The patients were subsequently followed up in the outpatient clinic for one year and contacted by phone every three months thereafter, while they were encouraged to come back if symptoms recurred.

RESULTS: Twenty-seven of the 31 patients completed the 8-wk treatment course, of them 23 (85.2%) achieved a complete remission indicated by resolution of symptoms and healing of fissure. Of the remaining four unhealed patients (14.8%), 2 opted to undergo lateral sphincterotomy and the other 2 to continue therapy for four additional weeks, resulting in healing of fissure. All the 25 patients with complete remission had a mean follow-up of 22.9 ± 14 (range 6-52) mo. Recurrence of symptoms occurred in four of these 25 patients (16%) who were successfully treated with an additional 4-wk course of 0.5% nifedipine ointment. Two of the 27 (7.4%) patients who completed the 8-wk treatment presented with moderate headache as a side effect of nifedipine.

CONCLUSION: Topical 0.5% nifedipine ointment, used as an agent in chemical sphincterotomy, appears to offer

INTRODUCTION

Anal fissure is a painful condition that affects a sizable majority of the population. The cause is controversial. However, current theories suggest that an initial traumatic tear fails to heal, because of the internal anal sphincter spasm, generating high pressure into the anal canal and leading to secondary local ischemia of the anal mucosa^[1,2]. Treatment aims at improving the blood supply to the ischemic area in order to facilitate healing by reducing, if necessary, the resting anal pressure, a function of the internal anal sphincter. Traditional surgical techniques for treatment include anal dilation and partial division of the internal sphincter, which may be complicated by incontinence^[3,4]. This significant complication has led to a search for alternative therapies for the treatment of anal fissure. The alternative options of "chemical sphincterotomy" include topical glyceryl trinitrate^[5,6], diltiazem^[7,8], botulinum toxin^[9,10], bethanechol^[7], indoramin^[11], and nifedipine^[12-14]. The aim of this study was to investigate the efficacy of local application of a new preparation of nifedipine ointment (0.5%) as an aggressive therapeutic modality in healing acute anal fissure and preventing its evolution to chronicity.

MATERIALS AND METHODS

Subjects

Thirty-one patients (10 males, 21 females, mean age 44.6 ± 11.9 years, range 19-69 years) with acute anal fissure

Table 1 Characteristics of the patients

Characteristic		%
Patients (n)	31	100
Mean age \pm SD (yr) (range)	44.6 \pm 11.9 (19-69)	
Gender ratio (M:F)	10:21	32.3:67.7
Location		
Posterior midline	27	87.1
Anterior midline	4	12.9
Pain	31	100
Bleeding	14	45.2
Mean follow-up \pm SD (mo) (range)	22.9 \pm 14 (6-52)	

participated in the study, from September 1999 to January 2005. The fissures were posterior in 27 patients (87%) and anterior in 4 patients (13%). Symptoms on presentation were anal pain in all patients (100%) and bright red bleeding per rectum in 14 patients (45.2%). The clinical characteristics of the patients are shown in Table 1.

Inclusion criteria included patients with acute anal fissure aged 18 years or older. Exclusion criteria were presumed or confirmed surgery, allergy to nifedipine, associated complications warranting surgery (abscess, fistula and cancer), Crohn's disease, tuberculosis ulcer, leukemic ulcer, HIV-related anal ulcer, cancer, and unwillingness of the patient to participate in the study. Concomitant first- to third-degree hemorrhoids were not considered an exclusion criterion.

Presence of acute anal fissure was considered if the patient presented with a history of anal pain at defecation for less than two months, that failed to resolve with conservative therapy consisting of stool softeners, high fiber diet and topical anesthetic creams prescribed by the general practitioner.

In the same time period we followed up 39 patients with acute anal fissure which was healed in only 8 patients after treatment with stool softeners and topical analgesics alone.

Methods

The study protocol was approved by the Ethics Committee of the Central Hospital of Thessaloniki, and all patients signed an informed consent. Anal manometry was performed in all patients before they entered the study and after two weeks of treatment. Manometric recordings and analysis of the tracings were conducted using a water-perfusion system. The anal canal pressure was recorded in cm of water by the stationary pull-through technique, using a water-filled micro-balloon and external transducer (PVD) perfusion equipment (Medtronic Inc, Bonn, Germany). The recording and analysis of the tracings were both made by a computerized system (8 channels polygraph ID, Medtronic Polygraph with Polygram 98, version 2.2 software, Medtronic, Inc., Minneapolis, MN, USA). Anal resting pressures were recorded, and the mean pressure was determined by the computer.

Topical nifedipine ointment regimen was prepared by a pharmacist diluting 100 mg of nifedipine in 20 g of yellow soft paraffin to produce a preparation of 0.5%. Patients were instructed to apply the ointment circumferentially 1

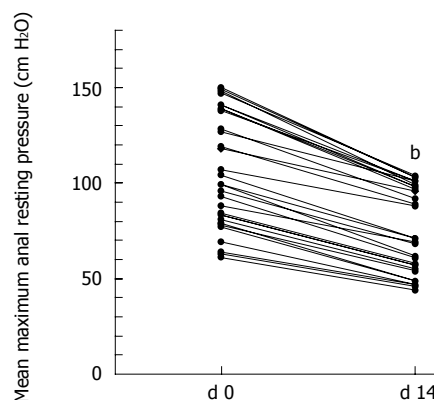


Figure 1 Mean maximal anal resting pressure after the application of nifedipine to the anus (^b $P < 0.0001$ d 14 vs d 0).

cm inside the anus, near the internal anal sphincter, every 8 h for 8 wk. The preparation was kept in a cool place, inside an opaque glass container with a screw top lid, and discarded 3 wk after formulation.

Patients were not prescribed stool softeners or topical analgesic creams during the treatment. A high-fiber diet was encouraged. The patients were followed up at 2, 4, and 8 wk in the outpatient clinic. During each visit, they were asked if they had pain and bleeding on defecation, and if they had headache, dizziness, flush, rash, and incontinence. Healing of acute anal fissure was set as the primary target of our study, and defined as epithelialization achieved on d 57 of therapy. If healing occurred after the initial 8-wk period, the patients were followed up in the outpatient clinic at 3, 6, and 12 mo, or sooner if recurrent symptoms occurred. Thereafter, the patients were contacted by telephone every three mo. If symptoms recurred, the patients were encouraged to call back, and given the option to undergo treatment with 0.5% nifedipine ointment for 4 consecutive wk, and the option of operation. If they selected the last option or if symptoms persisted after the additional 4 wk treatment, the patients were referred for lateral sphincterotomy.

Statistical analysis

The paired *t*-test was used to evaluate the impact of nifedipine application on the mean maximal anal resting pressure (MMRP). $P < 0.05$ was considered statistically significant. The analysis was performed using SPSS (Statistical Package for Social Sciences, version 10.0, Chicago, IL, USA).

RESULTS

The baseline MMRP was 139.6 (range 118-150) cm H₂O (normal range 60-160 cm H₂O) in the 10 male subjects and 90.3 (range 61-141) cm H₂O (normal range 43-142 cm H₂O) in the 21 female subjects. After topical application of 0.5% nifedipine ointment for 14 d, the MMRP was 100 (range 96-104) cm H₂O in males and 63.6 (range 44-102) cm H₂O in females (Figure 1).

The treatment outcomes are summarized in Figure 2. One patient continued to present severe anal pain in the first week of therapy and chose to be operated upon.

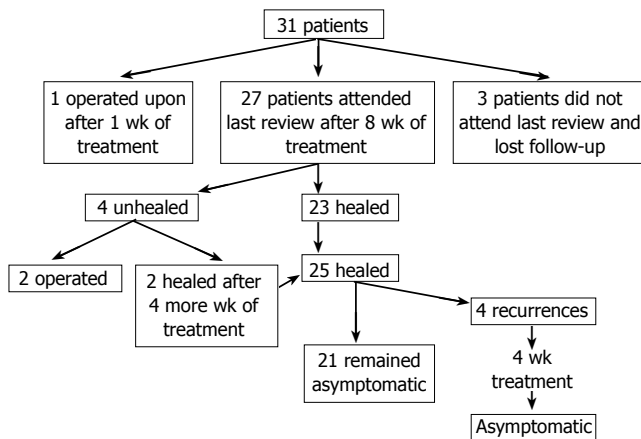


Figure 2 Outcome after aggressive treatment with 0.5% topical nifedipine ointment.

Twenty-seven of the 31 patients attended the last review at 8 wk, of them 23 (85.2%) had complete remission. The remaining 4 (14.8%) did not heal at 8 wk and were given the option of an additional course of nifedipine treatment or the option of lateral internal sphincterotomy. Two of these four patients opted for a further course of nifedipine therapy leading to complete remission, and the other two preferred to be operated.

The 25 patients who were successfully treated with 0.5% nifedipine ointment had a mean follow-up of 22.9 ± 14 (range 6–52) mo, of them 21 (84%) remained asymptomatic and 4 (16%) had recurrences that were successfully treated with an additional 4-wk course of treatment with 0.5% nifedipine ointment. It was not possible to predict healing from the initial manometric response to 0.5% nifedipine ointment.

Two patients with a history of migraine reported moderate headache while being treated with topical nifedipine, which was treated with paracetamol. No other side effects were reported.

DISCUSSION

Oral and topical calcium channel blockers (CCBs) have recently been shown to lower the anal resting pressure by relaxing the internal anal sphincter^[12–16]. The transport of calcium through the L-type calcium channels is important for the maintenance of internal anal sphincter tone^[17]. As opposed to glyceryl trinitrate, which reduces resting anal tone by releasing nitric oxide, nifedipine (a calcium channel blocker) reduces the tone and spontaneous activity of the sphincter by decreasing the intracellular availability of calcium^[17–19]. Oral administration of CCBs is associated with side effects such as hypotension and flushing, which may decrease compliance^[20]. Topical diltiazem and nifedipine are highly effective, achieving a healing rate of 67% for diltiazem and up to 95% for nifedipine^[8,12,13]. A recent randomized study by our group showed that the topical use of 0.5% nifedipine could achieve complete healing in 96.7% of the patients, not significantly different from the group treated with internal sphincterotomy^[21]. However, the problem with temporary “chemical sphincterotomy” is that after treatment the anal pressure

risks to pre-treatment levels, resulting in a high rate of recurrence. Recurrence has been reported in approximately 42% of patients treated with nifedipine^[22]. It should be noted, however, that the above healing and recurrence rates have been reported in patients with chronic anal fissures.

Acute anal fissure in adults is thought to precede chronic fissure and to be more analogous to pediatric anal fissure in its pathologic anatomy^[1]. It is commonly believed that if acute anal fissure is aggressively treated, it can be healed preventing the development of chronic fissure.

To the best of our knowledge, there is only one published work investigating the efficacy of nifedipine ointment in the treatment of acute anal fissure. In the study of Antropoli *et al.*^[12], 141 patients were treated topically with 0.2% nifedipine gel, every 12 h for 3 wk. The control group consisting of 142 patients received topical 1% lidocaine and 1% hydrocortisone acetate gel. Complete remission of acute anal fissure was achieved in 95% of the nifedipine-treated patients, as opposed to 50% of the controls. The study does not report the followed up patients. In contrast to the study by Antropoli *et al.*^[12], we used 0.5% (instead of 0.2%) nifedipine ointment, the period of treatment was 8 (instead of 3) wk and we included a relatively long-term follow-up of patients of 22.9 ± 14 (range 6–52) mo. The high rate of healing (85.2%) in our study after 8 wk of treatment is possibly related to the long duration of treatment, considering that the usual treatment for acute anal fissure ranges between 3 and 4 wk. This high healing rate may be attributed not only to the reduction of the anal canal pressure (significant in our patients) through the inhibition of the flow of calcium into the sarcoplasm of the internal anal sphincter, but also to the anti-inflammatory action of nifedipine. Experimental studies indicate that nifedipine has a modulating effect on the microcirculation^[23] and a local anti-inflammatory effect^[24], in addition to relaxation of the internal anal sphincter. From a further viewpoint, laser Doppler flowmetry has shown that the posterior area of the anoderm is less well perfused than other areas of the anoderm. It has been speculated that increased tone in the internal sphincter muscle further reduces the blood flow, especially at the posterior midline. Based on these findings, fissures are thought to represent ischemic ulceration^[25]. Since oxidative stress is believed to initiate and aggravate many diseases including peptic-ischemic ulcerations^[26,27], it could be speculated that nifedipine might promote acute fissure healing rate because of its additional free radical-scavenging properties as well as its cytoprotective and peptic ulcer healing-promoting actions^[28]. However, further studies are needed to elucidate these potential therapeutic properties of this drug in healing acute as well as chronic anal fissures.

A high fiber diet by itself or in addition to topical ointments consists of a part of acute anal fissure treatment. Since our study included patients with acute anal fissures, which did not respond to conservative therapy consisting of stool softeners, high fiber diet and topical anesthetic cream, it is impossible to attribute the success of treatment to high fiber diet which we encouraged the patients to continue.

It should be emphasized that the two unhealed patients after 8 wk of treatment and all recurrences during the follow-up were successfully treated with an additional 4-wk course of topical 0.5% nifedipine ointment. The main limitation of this study is the lack of a placebo group. Although CCBs have not been directly compared in any analysis with an arm termed as placebo, there are 2 studies comparing nifedipine with either hydrocortisone^[13] or lidocaine^[12]. A marked advantage of nifedipine over substances currently thought to be the equivalent of placebo has been reported, with a healing rate of approximately 35% in the placebo groups^[13]. Our study has shown a healing rate of 85.2%, significantly higher than the previously reported rate in placebo groups (35%).

Another drawback in our study suggests that treatment of acute anal fissure with 0.5% nifedipine ointment is the length of treatment (8 wk), which is generally considered to be longer than the typical length (usually 3-4 wk). Although this extended length of treatment may increase the percentage of non-compliant patients, only 3 out of the 31 patients (10%) did not complete the 8-wk treatment in our study. This non-compliance percentage is comparable to that of other studies^[7,8,12,13].

An interesting finding of our study is the fact that, despite the applied dosage of nifedipine (0.5%) was at least twice as high as in previous studies^[12,13], there was no increase in adverse effects. Only two patients (7.4%) presented with moderate headache, which was relieved with paracetamol.

In conclusion, topical application of 0.5% nifedipine ointment might be effective both in treating acute anal fissure and in preventing its evolution to chronicity.

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Interrelationship between chromosome 8 aneuploidy, *C-MYC* amplification and increased expression in individuals from northern Brazil with gastric adenocarcinoma

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chromosome 8 ploidy and the site, stage or histological type of the adenocarcinomas. *C-MYC* high amplification, like homogeneously stained regions (HSRs) and double minutes (DMs), was observed only in the intestinal-type. Structural rearrangement of *C-MYC*, like translocation, was observed only in the diffuse type. Regarding *C-MYC* gene, a significant difference ($P < 0.05$) was observed between the two histological types. The *C-MYC* protein was expressed in all the studied cases. In the intestinal-type the *C-MYC* immunoreactivity was localized only in the nucleus and in the diffuse type in the nucleus and cytoplasm.

CONCLUSION: Distinct patterns of alterations between intestinal and diffuse types of gastric tumors support the hypothesis that these types follow different genetic pathways.

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Key words: Chromosome 8 aneuploidy; *C-MYC* amplification; Immunostaining; Gastric adenocarcinoma

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Abstract

AIM: To investigate chromosome 8 numerical aberrations, *C-MYC* oncogene alterations and its expression in gastric cancer and to correlate these findings with histopathological characteristics of gastric tumors.

METHODS: Specimens were collected surgically from seven patients with gastric adenocarcinomas. Immunostaining for *C-MYC* and dual-color fluorescence *in situ* hybridization (FISH) for *C-MYC* gene and chromosome 8 centromere were performed.

RESULTS: All the cases showed chromosome 8 aneuploidy and *C-MYC* amplification, in both the diffuse and intestinal histopathological types of Lauren. No significant difference ($P < 0.05$) was observed between the level of

INTRODUCTION

Gastric cancer is the third most frequent type of cancer in the world^[1]. In Northern Brazil, the State of Pará presents a high incidence of this neoplasia type and its capital, Belém, is ranked eleventh in number of gastric cancers per inhabitant among all cities in the world with cancer records^[2]. Food factors may be related to the high incidence of this neoplasia in Pará, specially high consumption of salt-conserved food, reduced use of refrigerators and little consumption of fresh fruit and

vegetables^[3].

Gastrointestinal tract tumors are notorious for being difficult to analyze by standard cytogenetic techniques^[4-8]. Fluorescence *in situ* hybridization (FISH) technique with specific DNA probes allows rapid detection of chromosome aberrations in tumor interphase nuclei. In FISH studies, numerical aberrations in chromosomes 1, 7, 8, 9, 17, 20, X and Y are common^[9-12]. Chromosome 8 abnormalities are frequent, not only in gastric neoplasias, but also in several types of hematopoietic proliferations and solid tumors^[13-15].

Among the genes found on chromosome 8, *C-MYC*, located at 8q24, has been the most studied. *C-MYC* gene is a regulator of cell cycle and plays a major role in control of cell growth, differentiation, apoptosis and neoplastic transformation^[16]. *C-MYC* gene overexpression is a frequent alteration and has been described in several types of human cancer^[17-19]. An increased *C-MYC* gene expression has been found in gastric neoplasias^[20-23].

The aim of this study was to investigate chromosome 8 numerical aberrations, *C-MYC* oncogene alterations and its expression in gastric cancer samples from the State of Pará, using the FISH technique and immunohistochemistry. Possible correlations between these findings and histopathological characteristics were also evaluated.

MATERIALS AND METHODS

Cases studied

Seven samples of primary tumors submitted to surgical resection were obtained from male patients in Pará State João de Barros Barreto University Hospital (HUIBB). Patients' ages and tumors' anatomical sites were obtained from tumor registries (Table 1). The patients had never been submitted to chemotherapy or radiotherapy prior to surgery, nor had they any other diagnosed cancer. Genetic study of samples was approved by the Ethics Committee of HUIBB. A fraction of each sample was used for routine histopathological diagnosis according to Laurén's classification^[24].

Immunostaining

For antigen retrieval, deparaffinized sections (5 µm) were pretreated by heating in a microwave oven in citrate buffer 10 mmol/L, pH 6.0 for 20 min. After cooling, sections were immersed in PBS containing 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections were then incubated in a humid chamber overnight at 4°C with primary antibody C-MYC (clone 9E10.3; dilution 1:100). After rinsing with PBS, slides were incubated with secondary antibody followed by streptavidin-biotin-peroxidase complex, both for 30 min at room temperature with a PBS wash between each step. Slides were visualised with diaminobenzidine-H₂O₂ and counterstained with Harry's hematoxylin.

Fluorescence in situ hybridization

Tumor samples from all patients were processed for cytogenetic study as described previously^[25]. FISH assay

was performed on slides with cells fixed in methanol/acetic acid. A directly labeled dual-color probe was used for chromosome 8 alpha-satellite region (8q11) and for *C-MYC* gene region (8q24). Slides were washed in 2 × saline sodium citrate solution (SSC) and dehydrated in 70%, 80% and 95% ethanol, respectively. Samples were then denatured with 70% formamide/2 × SSC (pH 7.0) at 70°C for 2 min and transferred to an iced ethanol (-20°C) series at 70%, 80% and 95%. Probes were denatured at 96°C for 5 min. Then, 10 µL was applied onto the slide under a glass coverslip. *In situ* hybridization occurred at 37°C in a moist chamber overnight. Post-hybridization washings were done, and nuclei were counterstained with DAPI/antifade. Molecular cytogenetic analysis was carried out under an Olympus BX41 fluorescence microscope with triple DAPI/FITC/TRICIT filter and an Applied Spectral Imaging image analysis. For each sample, 200 interphase nuclei were analyzed. To avoid misinterpretation due to technical error, normal lymphocyte nuclei and normal gastric tissue were used as a control.

Statistical analysis

For statistical evaluation, chi-square test was used. $P < 0.05$ was taken as significant.

RESULTS

All seven samples studied were histologically classified as gastric adenocarcinomas, 2 of them were diffuse type and 5 were intestinal type, according to Laurén's classification (Table 1).

C-MYC was expressed in all cases. *C-MYC* immunoreactivity was localized in nucleus and cytoplasm of diffuse type samples, but only in nucleus of intestinal type (Figure 1A and 1B).

In peripheral blood lymphocytes, two signals were observed in 98.5% of analyzed nuclei for chromosome 8 probe and 99.5% for *C-MYC* gene probe. Normal stomach tissue showed two signals in 96% of analyzed nuclei for chromosome 8 probe and 98% for *C-MYC* gene probe. All gastric adenocarcinoma cases showed numerical increase of chromosome 8 and *C-MYC* gene (Table 1).

Chromosome 8 trisomy was detected in all cases, varying from 19% (case 3) to 33% (case 1), and chromosome 8 tetrasomy (observed in all cases) varied from 1% (case 4) to 18% (case 3). Five signals for chromosome 8 were observed in 6 cases (85.7%) and the highest frequency was found in case 7 (4%). Six or more signals were observed in 1 case (case 7).

Presence of 6 or more signals for *C-MYC* gene was considered as an intermediary degree of amplification, whereas the cases which presented double minutes (DMs) and/or homogeneously stained regions (HSRs) were classified as presenting a high degree of gene amplification. Cells with more than six signals were found in 5 cases (71.42%). The frequency of cells with high amplification (Figure 1C) of this gene varied from 1% (case 4 and 5) to 6% (case 7) (Table 1). Thus, all intestinal type cases and none diffuse type presented intermediary and high amplification of *C-MYC*.

Table 1 Number of signals by percentage of analyzed nuclei

Case	Age (yr)	Origin	HT	UICC	Immunostaining	Probe	Percentage of nuclei						
							Number of signals						
							1	2	3	4	5	≥ 6	HA
1	77	Antrum	Intestinal	T2N1M0	Nuclear	C-MYC	0	34	23	25	14	2	2
						Chrom.8	0	58	33	9	0	0	-
2	48	Antrum	Intestinal	T4N0M0	Nuclear	C-MYC	0	35	10	20	29	2	4
						Chrom.8	0	70	21	6	3	0	-
3	74	Antrum	Diffuse	T1N0M0	Nuclear/Cytoplasmatic	C-MYC	1	38	29	30	2	0	0
						Chrom.8	2	59	19	18	2	0	-
4	74	Antrum/body	Intestinal	T4N0M0	Nuclear	C-MYC	0	40	12	28	17	2	1
						Chrom.8	0	63	25	1	1	0	-
5	41	Antrum	Intestinal	T2N0M0	Nuclear	C-MYC	0	36	18	30	13	2	1
						Chrom.8	4	61	30	4	1	0	-
6	56	Antrum/body	Diffuse	T2N1M0	Nuclear/Cytoplasmatic	C-MYC	0	31	38	17	14	0	0
						Chrom.8	3	60	32	5	1	0	-
7	55	Antrum	Intestinal	T4N2M1	Nuclear	C-MYC	2	48	27	7	7	3	6
						Chrom.8	2	54	27	5	4	8	-
Control	77	Stomach tissue	-	-	Without staining	C-MYC	2	98	0	0	0	0	0
						Chrom.8	1	96	3	0	0	0	-
Control	37	Lymphocytes	-	-	Without staining	C-MYC	0.5	99.5	0	0	0	0	0
						Chrom.8	1	98.5	0.5	0	0	0	-

HT: Histological type; UICC: Union Internationale Contre le Cancer; HA: High amplification.

Rearrangements between *C-MYC* gene and chromosome 8 centromere were discriminated by evident separation of the two signals. In tumor samples, translocation was observed between *C-MYC* gene and another chromosome in case 3 (2%) and case 6 (4%), both of them of diffuse type (Figure 1D).

Analyzing the total number of signals for *C-MYC* gene and for chromosome 8 centromere, it was found that the number of signals for the gene was greater than the number of signals for chromosome 8 ploidy in all cases studied.

No significant difference ($P < 0.05$) was observed between chromosome 8 ploidy level and adenocarcinoma site, stage or histological type. Regarding *C-MYC* gene, a significant difference was observed between the two histological types. This difference was due to the presence of HSRs and/or DMs in Laurén's intestinal type, where multiple (uncountable) signals were found per cell.

DISCUSSION

Gastric cancer is one of the most common neoplasias and both environmental and genetic factors contribute to its occurrence^[26]. The present study used interphase dual-color FISH with direct fluorescent labeling for the chromosome 8 centromere/*C-MYC* gene and compared the copy number observed in 7 gastric adenocarcinoma samples with *C-MYC* expression by immunohistochemistry.

C-MYC amplification has been reported in a small percent of gastric carcinomas^[21,27,28]. *C-MYC* expression was shown to be more frequent in diffuse than in intestinal type gastric cancer cells, and more frequent in gastric adenocarcinoma than in adenoma^[29,30]. In the present study, *C-MYC* was expressed in all cases. *C-MYC* immunoreactivity was localized in the nucleus in intestinal-type and in cytoplasm and nucleus in diffuse type. More samples need to be investigated, in order to clarify if this

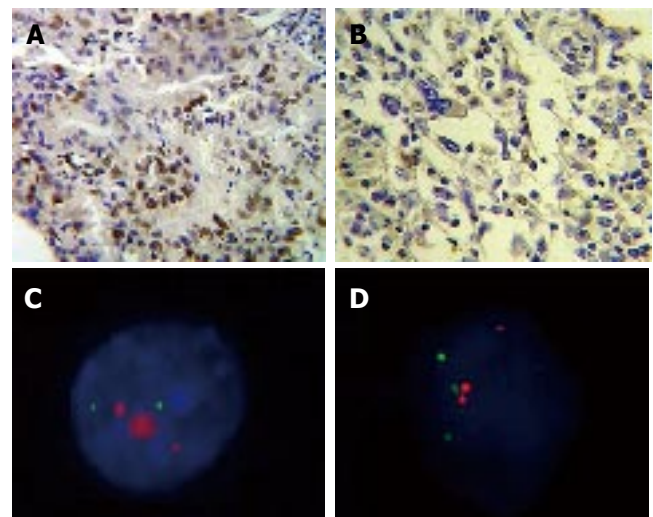


Figure 1 Cells submitted to immunohistochemistry and FISH techniques. **A:** Infiltrating gastric adenocarcinoma of intestinal type shows intense nuclear marcation for *C-MYC*, $\times 400$; **B:** Gastric adenocarcinoma of diffuse type nuclear marcation and cytoplasmic light marcation for *C-MYC*, $\times 400$; **C:** Interphase nuclei presenting *C-MYC* high amplification (red) and chromosome 8 (green); **D:** Interphase nuclei presenting chromosome 8/*C-MYC* rearrangement.

immunostaining differences exist.

Our laboratory has previously observed the presence of chromosome 8 trisomy in all 16 cases studied by direct chromosome analysis and centromeric FISH^[31] and in 60% of analyzed cells of ACP01 gastric adenocarcinoma cell line^[32]. The gain of signals for the two probes analyzed in all samples in the present study corroborates with these data.

Panani *et al.*^[9] analyzed 33 gastric tumor samples by FISH, using a chromosome 8 alpha-satellite probe. Numerical aberrations in this chromosome were observed in 62.16% of studied samples, in which trisomy was detected in 43.24%, tetrasomy in 10.81% and monosomy

in 8.10%. Our results confirmed that chromosome 8 trisomy is a common biological phenomenon in adenocarcinoma of stomach and can be used as a gastric mucosa malignancy marker. In our study, 100% of samples presented a gain of chromosome 8 as a clonal alteration.

Chromosome 8 numerical abnormalities, in which *C-MYC* is located, are suggested to be an important mechanism in *C-MYC* copy number increase. Xia *et al*^[5] suggested that chromosome 8 trisomy, associated or not with other chromosomal aberrations, could occur even in less advanced stages of the disease, possibly prior to the occurrence of metastases.

Kitayama *et al*^[12] analyzed interphase nuclei of 51 gastric cancer cases from pathology archives using 18 centromeric probes, including chromosome 8 probe, and a probe for *C-MYC* gene. They observed chromosome 8 numerical abnormalities in 56.9% of samples, which placed them among the most frequent alterations; in 12 cases, a *C-MYC* gain was observed and all of them presented chromosome 8 gain. Amplification of this oncogene was also reported in other FISH studies in gastric neoplasias^[33-35].

C-MYC oncogene seems to be fundamental in the oncogenesis process. Thus, increased *C-MYC* allele number is directly related to the degree of tumor aggressiveness, considering that the greater the gene copy number, the higher its level of expression. This gene was amplified in all samples studied, but without numerically accompanying chromosome 8 ploidy; that is, in all cases there were more *C-MYC* gene alleles than this chromosome copies.

In another study conducted in our laboratory^[36], 90.9% of cases presented intermediary amplification, 60% of which presented DMs and HSRs. It seems that, as our sample presented chromosome 8 gain as a clonal characteristic, *C-MYC* amplification is a later step, a consequence of carcinogenesis clonal expansion.

We have previously demonstrated, by comparative genomic hybridization, gains at region 8q24.1 (38.1%), which were found exclusively, in all intestinal type adenocarcinomas with systemic metastasis (M1). Results from this work also showed the highest amplification level in gastric cancer of intestinal type. *C-MYC* locus amplification may be an aggressiveness predictor in intestinal type gastric cancer, playing an important role in its development and progression. These results should provide useful information for developing more effective strategies in management of gastric cancer^[37].

Stamouli *et al*^[25] studied, by multicolor FISH, two primary gastric adenocarcinoma cases: a well-differentiated intestinal-type and a poorly differentiated diffuse type. The intestinal-type exhibited few structural abnormalities, in contrast to the diffuse type. In our analysis, all diffuse type cases presented at least four cells with translocation. It seems that this histological type is more susceptible to chromosomal rearrangements than intestinal type.

Correa^[38] suggested that the intestinal type fits the multiple-step process. Thus, it is plausible that the intestinal type presents a greater number of DMs and/or HSRs than the diffuse type. Our findings support that these two histological types follow different genetic tumorigenesis mechanisms^[39]. Moreover, it could be observed that translocations were restricted to diffuse

type. This result can be explained by the fact that gene amplification is not necessarily associated or required for its overexpression. Leukemias, lymphomas and sarcomas commonly elicit specific balanced translocations which mediate proto-oncogenes activation, by their juxtaposition with promoter sequences or generating gene fusion^[40].

Enhanced *C-MYC* protein expression contributes to almost every aspect of tumor cell biology. Although the ability of *C-MYC* to drive unrestricted cell proliferation and to inhibit cell differentiation had been well recognized, a recent work showed that deregulated *C-MYC* expression can drive cell growth and vasculogenesis, reduce cell adhesion, and promote metastasis and genomic instability. On the other hand, *C-MYC* loss not only inhibits cell proliferation and cell growth, but can also accelerate differentiation, increase cell adhesion and lead to an excessive response to DNA damage. Studies in animal models suggest that *C-MYC* may be a target for human cancer treatment, but it is still unknown whether such drugs will be useful^[41].

The alterations found in this study have been described in the literature, even though their frequency was higher in our sample. Considering that external factors, such as eating habits and other environmental agents, have a direct influence on the development of this neoplasia, many genetic alterations may be regional characteristics of a given population.

Based on our findings we could also affirm that gastric adenocarcinomas of differing histopathological features are associated with distinct patterns of genetic alterations, suggesting that they evolve through different genetic pathways.

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RAPID COMMUNICATION

Relationship between antioxidant capacity and oxidative stress in children with acute hepatitis A

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Abstract

AIM: To investigate in children with acute hepatitis A. According to our knowledge, there are no data about the blood levels of malondialdehyde (MDA, an indicator of oxidative stress) and nonenzymic antioxidants in children with acute hepatitis A.

METHODS: Whole blood MDA and reduced glutathione (GSH), serum β -carotene, retinol, vitamin E and vitamin C levels were studied in 19 (10 females, 9 males) children with acute hepatitis A and in 29 (13 females, 16 males) healthy control subjects.

RESULTS: There was a statistically significant difference between patients and controls for all parameters ($P < 0.05$). Lipid peroxidation marker MDA was significantly elevated ($P < 0.001$), while antioxidants β -carotene, retinol and GSH were significantly decreased (all $P < 0.001$) in patients compared to healthy subjects. In addition, α -tocopherol and ascorbic acid levels were significantly lower in patients when compared to age and sex matched controls ($P < 0.05$, $P < 0.01$, respectively).

CONCLUSION: Our study shows that hepatitis A virus induces oxidative stress in children with hepatitis A. This finding could be taken into consideration to improve the therapeutic approach in acute hepatitis A.

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Key words: Antioxidant; Oxidative stress; Hepatitis A; Child

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INTRODUCTION

Acute viral hepatitis is one of the most common infectious diseases while hepatitis A is the most prevalent form of acute viral hepatitis in many parts of the world. In developing countries located in Africa, Asia, and Latin America, seroprevalence rates approach 100% and most infections occur by 5 years of age. By contrast, seroprevalence rates in the USA, Western Europe, and in several Mediterranean countries, have been falling during the past few decades^[1]. Viral hepatitis is also a major health problem in Turkey. The most important causes of spreading the disease are low levels of socioeconomic status and poor hygiene conditions, particularly in Eastern Turkey^[2,3]. Hepatitis A mostly occurs in the context of community-wide epidemics during which infection is transmitted from person to person by the fecal-oral route^[4]. The highest rates of disease are seen among children and young adults, and asymptomatic infection among young children is common^[5].

Mitochondria and cytochrome P450 enzymes are the main sources of reactive oxygen species (ROS) in hepatocytes acutely and/or chronically exposed to a "toxic" injury (e.g., environmental drugs, alcohol, therapeutical drugs, viruses). ROS also derive from Kupffer and inflammatory cells, in particular neutrophils^[6]. Oxygen-containing free radicals (such as the hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxyxynitrite) are highly reactive species, capable in the nucleus and in the membranes of cells of damaging biologically relevant molecules such as DNA, protein, carbohydrates, lipids^[7] and are produced in physiological and pathological conditions in living organisms. Lipid peroxidation (LPO) is an autocatalytic process which is a common consequence of cell death. This process may cause peroxidative tissue damage in inflammation, cancer, toxicity of xenobiotics and aging. Polyunsaturated fatty acids of the membrane are peroxidized by free radical-mediated reactions. Malondialdehyde (MDA) is one of the end-products in

the LPO process^[8,9]. Oxidative stress is a reflection of excess intracellular concentrations of ROS^[10] and one of the important indicators of cellular damage. Antioxidants transform free radicals into less reactive species, thereby limiting their toxic effects. There are several endogenous antioxidant systems to deal with the production of ROS. These systems can be divided into enzymic and nonenzymic groups. The enzymic subgroup includes superoxide dismutase, catalase and glutathione peroxidase. The nonenzymic group includes a variety of biologic molecules, such as vitamins E, A, and C and reduced glutathione (GSH)^[11].

According to our knowledge, there are no data concerning the effects of oxidative stress on blood nonenzymic antioxidant status in children with hepatitis A. Hence, in the present study, we measured lipid peroxidation levels and antioxidant status such as fat-soluble (vitamin A and vitamin E) and water-soluble (vitamin C and GSH) in children with hepatitis A.

MATERIALS AND METHODS

The investigation included 19 children with acute hepatitis A and 29 control subjects who were admitted to Yüzüncü Yıl University, Faculty of Medicine Department of Pediatrics. The definition of the patients was based on clinical, biochemical and serological criteria. The determination of hepatitis A was made by a pediatrician after taking a detailed history and examining the children for signs of infection. The clinical and biochemical criteria are acute illness compatible with hepatitis and serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels greater than 20-50 times the normal upper limit. However, the diagnosis of hepatitis A was confirmed by serologic tests (IgM and total anti-HAV antibodies were detected by enzyme-linked immunosorbent assay). The control group consisted of healthy children with normal physical examination. None of them had a history of recurrent or recent infection.

Parents of the children, who were enrolled in the study, were informed about the purpose of the study and their consent was obtained. In the present study, whole blood MDA and GSH, and serum β -carotene, retinol, α -tocopherol, ascorbic acid, ALT, AST and total bilirubin levels were measured in all of the subjects. Fasting venous blood samples for the biochemical analysis were taken from each person and transferred to heparinized and normal tubes. Whole blood was collected into heparinized tubes and whole blood MDA and GSH levels were studied on the same day of admission. MDA, which is an important indicator of lipid peroxidation, was determined by spectrophotometry of the pink-colored product of the thiobarbituric acid-malondialdehyde complex formation^[12]. Whole blood GSH concentration was measured by a spectrophotometric method^[13]. Serum was obtained by centrifugation at 2000 *g* for 10 min, of blood samples taken without anti-coagulant which was allowed to clot for 30 min at room temperature, and stored at -20°C until analysis date. Hemolysed samples were excluded. To avoid isomerization of vitamin A and vitamin E, blood samples

Table 1 Serum antioxidant vitamins and whole blood MDA and GSH levels in the patient and control groups (mean \pm SD)

	Acute Hepatitis A (<i>n</i> = 19)	Control (<i>n</i> = 29)	<i>P</i>
MDA (nmol/mL)	1.92 \pm 0.14	1.11 \pm 0.11	< 0.001
GSH (mg/dL)	3.89 \pm 1.59	34.38 \pm 1.41	< 0.001
β -carotene (μ g/dL)	10.52 \pm 1.19	17.44 \pm 1.78	< 0.001
Retinol (μ g/dL)	20.87 \pm 1.42	32.81 \pm 2.13	< 0.001
α -tocopherol (mg/dL)	0.56 \pm 0.31	0.71 \pm 0.25	< 0.05
Ascorbic acid (mg/dL)	0.88 \pm 0.17	1.29 \pm 0.18	< 0.01

P values were calculated using the Student's *t*-test. MDA: Malondialdehyde; GSH: Reduced glutathione; Retinol: Vitamin A; α -tocopherol: Vitamin E; Ascorbic acid: Vitamin C.

were protected from light as soon as they were drawn. The levels of β -carotene at 425 nm and vitamin A (retinol) at 325 nm were detected after the reaction of serum: ethanol: hexane at the ratio of 1:1:3, respectively^[14]. Vitamin E (α -tocopherol) was analyzed colorimetrically with 2,4,6-tripridyl-s-triazin and ferric chloride after extraction with absolute ethanol and xylene^[15]. Serum vitamin C (ascorbic acid) level was determined after derivatization with 2,4-dinitrophenylhydrazine^[16]. Serum ALT, AST and total bilirubin levels were measured in an autoanalyzer (Roche). The same parameters were also studied in the control subjects.

The results are expressed as mean \pm SD. Student's *t*-test was used to compare the mean values of different biochemical parameters between hepatitis A and control groups. In all data analysis, a value of *P* < 0.05 was considered statistically significant.

RESULTS

Our study included 19 (53% female, 47% male) children with acute hepatitis A and 29 (45% female, 55% male) healthy control subjects. The age ranged from 2-9 years (4.42 \pm 1.77 years) and 2-12 years (4.89 \pm 2.22 years) in the study and control groups, respectively. The two groups were comparable in terms of age and gender (*P* > 0.05). Serum enzyme markers of hepatic injury (ALT, AST, and total bilirubin) were significantly raised in the study group. The ALT level ranged from 907 to 4051 IU/L (1869.22 \pm 939.3 IU/L, normal values \leq 40 IU/L), the AST level ranged from 352 to 4690 IU/L (1827.11 \pm 1251.5 IU/L, normal values \leq 40 IU/L) and the total bilirubin level ranged from 1.15 to 8.05 mg/dL (4.98 \pm 1.4 mg/dL, 0.2-1.2 mg/dL) in the patients' group.

Whole blood MDA and GSH levels and serum β -carotene, retinol, α -tocopherol and ascorbic acid levels in the patient group and healthy subjects are presented in Table 1. As seen in the Table, there were statistically significant differences between the groups for all parameters (*P* < 0.05). MDA (as indicator of the lipid peroxidation) was markedly increased in the children with acute hepatitis A when compared to the control group (*P* < 0.001). However, nonenzymic antioxidant status such as fat-soluble (vitamin A and vitamin E) and water-soluble (vitamin C and GSH) were found to be decreased in the

study group. Serum β -carotene and retinol, and whole blood GSH levels were significantly lower in children with hepatitis A than in the control group (all $P < 0.001$). Nevertheless, serum α -tocopherol and ascorbic acid levels were also significantly decrease in patient group ($P < 0.05$, $P < 0.01$, respectively).

DISCUSSION

The normal liver is a well equipped organ in terms of either enzymic or nonenzymic antioxidants although most of the hepatic antioxidant defenses are essentially confined to parenchymal cells. Kupffer cells, hepatic stellate cells or endothelial cells are potentially more exposed or sensitive to oxidative stress-related molecules. Published experimental evidence clearly indicates that hepatic as well as plasma antioxidant defenses (in particular, GSH and α -tocopherol) are often significantly decreased in several liver disease^[17,18].

Oxidative stress is a physiologic consequence of aerobic metabolism. The intermediate components formed in aerobic organisms, such as superoxide and hydrogen peroxide, lead to the further production of ROS that can oxidize membrane lipids and disrupt metabolic processes. GSH is essential for protection against these toxic products of oxygen metabolism, especially as a substrate for GSH peroxidases^[19]. Inflammation, oxidative stress and medications that are detoxified through GSH consuming pathways will all result in GSH loss and excessive cysteine catabolism^[20].

GSH depletion in lymphoid cells may interfere with the immunological mechanisms involved in viral clearance, thus facilitating viral replication and enhancing liver damage because of the oxidative stress. Lymphocyte activation *in vitro* can be inhibited completely by decreasing GSH by 10% to 40%^[21] and Barbaro *et al*^[22] reported the depletion of GSH in chronic hepatitis C (CHC) patients was associated with a mean 12.7% reduction of peripheral blood mononuclear cells cytotoxic activity compared to controls^[22].

ROS are well characterized mediators of cell and tissue injury. Generally, cells defend themselves from ROS and other toxic oxygen species by a variety of mechanisms including nonenzymic and enzymic defence systems. An ineffective scavenging capacity of antioxidant systems may play a relevant role in determining the degree of oxidative stress^[23-25].

The increase in serum MDA concentration in CHC patients may very well fit in with the recently reported glutathione depletion observed in hepatic tissue, plasma, and peripheral blood mononuclear cells of CHC patients^[26]. Hepatitis C virus (HCV) infection is associated with reduced GSH levels in both plasma and erythrocytes known to be the main intracellular mechanism against oxidative stress, and this depletion appears to be related to the activity of the liver disease^[26,27]. Likewise, the present study also reports an increased MDA and decreased GSH levels in the acute hepatitis A patients.

The decreased content of cellular and plasma GSH, most likely mediated by HCV, or in the present paper's case by hepatitis A virus, may render biological structures

more susceptible to oxidative attack and this condition may expose circulating lipids and proteins to oxidative modifications with consequent loss of their biological functions. Several authors' findings are consistent with each other, in which it is reported that a reduction of hepatic GSH stores might be partly responsible for the cytopathic effect induced by HCV^[28,29]; it is also conceivable that the observed GSH depletion in lymphoid cells^[26] might interfere with the immunological mechanism involved in viral clearance, thus facilitating viral replication and enhancing, with time, liver damage through a greater oxidative stress^[30].

Free oxygen radicals take part in pathogenesis of chronic hepatitis of B and C type in children as they decrease the antioxidant barrier efficiency diminishing catalase and superoxide dismutase levels^[31]. In the same manner, it is likely that free oxygen radicals can take role in the pathogenesis of the acute hepatitis A since antioxidant vitamin levels were decreased in our study. As mentioned above, the reason might be the altered antioxidant capacity because of the decreased antioxidant vitamins in blood.

As expected from the other hepatitis cases, in acute hepatitis A, nonenzymic antioxidant levels such as GSH and β -carotene, retinol, vitamin E, vitamin C were found to have decreased in this study.

There are several possible explanations for the low serum retinol levels seen in chronic liver disease. Defective synthesis of retinol binding protein prevents the customary mobilization of retinol from the liver to the periphery. This would suggest that retinol exerts its antiproliferative effect in a paracrine or systemic manner rather than by an immediate local effect. Another possibility is that the absorption of dietary retinol is impaired, which may also explain why levels are even lower in patients with cholestatic liver disease. Whilst other groups have suggested that serum retinol levels are a reflection of nutritional status^[32], the other authors did not report any association between decreasing body mass index and lower retinol levels within the cohort of patients studied. In conformity with our results, von Herbay *et al*^[33] showed that vitamin E plasma levels were significantly lower ($P < 0.01$) compared to the control group. They suggest that the decreased vitamin E levels in patients with acute or chronic viral hepatitis with high activity of disease may be due to free radical-mediated liver injury.

Vitamin C, the major water-soluble antioxidant, is beneficial in reducing oxidative stress, but is harmful depending on the sensitive balance of its concentration. Ascorbic acid has been shown to efficiently scavenge superoxide, hydrogen peroxide, hypochloride, hydroxyl radicals, and peroxy radicals^[34] and to restore the antioxidant properties of fat-soluble α -tocopherol, therefore; it interrupts the radical chain reaction of lipid peroxidation^[35]. In this study, vitamin C levels were found to be significantly lower in the hepatitis A group than in the controls (Table 1). To our knowledge, there is no data concerning the levels of serum vitamin C in children with hepatitis A. The reduction in serum concentration of vitamin C might be due to increased rate of ascorbic acid oxidation in oxidative stress.

In conclusion, this study shows that hepatitis A virus

induces oxidative stress in children with hepatitis A. This finding could be taken into consideration to improve the therapeutic approach in acute hepatitis A.

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RAPID COMMUNICATION

Evaluation of the use of rapid urease test: Pronto Dry to detect *H pylori* in patients with dyspepsia in several cities in Indonesia

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Abstract

AIM: To evaluate Pronto Dry examination in patients with dyspepsia.

METHODS: The study was conducted in patients with dyspepsia who underwent endoscopic examination in several endoscopic centers of several cities in Indonesia from January 2003 until April 2004. Biopsies for histopathologic examination were fixed with formalin and sent to Histopathologic Department to be analyzed and confirm the presence of *H pylori* infection. If *H pylori* was found positive, the density was calculated semi quantitatively. Histopathologic examination from gastric biopsy samples was interpreted based on the updated Sydney system classification.

RESULTS: Of 550 patients, 309 (56%) were male and 241 (44%) were female with ages ranging from 15 to 82 years. Mean age was 44.98 ± 14.46 years. Mean age of male patients was 44.35 ± 13.85 years and mean age of female patients was 45.78 ± 15.19 years. Evaluation of endoscopic results showed gastric ulcer in 36 cases (6.5%) and duodenal ulcer in 20 cases (3.6%). Normal endoscopic finding was found in 45 cases (8.2%) and

minimal disorder of gastritis and duodenitis were found in 246 cases (44.7%). One case of gastric cancer was identified. Of 56 cases which were positive based on the criteria used, 39 patients were positive with Pronto Dry and 17 patients were negative with Pronto Dry. Overall sensitivity and specificity of Pronto Dry were 69.7% and 95.7% respectively. Positive predictive value was 66.1% and negative predictive value was 96.4% and overall accurate rate was 92.9%.

CONCLUSION: Pronto Dry seems promising as a diagnostic tool to detect *H pylori* more rapidly and accurately.

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Key words: Rapid urease test; Pronto Dry; *H pylori*; Dyspepsia

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INTRODUCTION

H pylori is a microaerophilic, gram negative, slowly growing, and pathogenic bacterium which produces urease enzyme^[1]. *H pylori* may cause chronic gastritis and predispose to gastric and duodenal ulcer. Recently, *H pylori* has been classified as gastric carcinogen class 1^[2,3].

Diagnosis of *H pylori* may be made by invasive or non invasive methods. During the invasive method patients would undergo endoscopic examination and gastric tissue biopsy. Samples and biopsies are examined to detect the presence of *H pylori* by histopathologic examination, rapid urease test or culture^[4,5]. Culture examination is the gold standard test. Culture examination is not simple because transport media and biopsy samples for observation of the growth of the bacteria need special preparation procedures.

Non invasive methods include the rapid urease test, serologic and *H pylori* examination of the feces (*H pylori* stool antigen)^[6]. Rapid urease test is frequently done in

endoscopic centers in patients with dyspepsia to detect the presence of *H pylori*. This method is rapid and easy to perform in the endoscopic room. Currently, there are various kinds of rapid urease test in the market such as CLO, MIU or Pyloric test and a recent one called Pronto Dry. The advantage of this method is that it is not necessary to keep the sample in the refrigerator, a rapid result may be obtained only in 1 h, and it is easy to read the color changes. Previous studies have shown this test has good sensitivity and specificity^[7,8].

The aim of the present study was to evaluate Pronto Dry examination in patients with dyspepsia in several endoscopic centers in Indonesia including Jakarta, Bandung, Surabaya, Denpasar, Yogyakarta and Medan.

MATERIALS AND METHODS

Research subjects

The study was conducted in patients with dyspepsia who underwent endoscopic examination in the endoscopic centers of several cities in Indonesia which were as follows: RSCM Jakarta, RS Hasan Sadikin Bandung, RS Sardjito Yogyakarta, RS Soetomo Surabaya, RS Adam Malik Medan, and RS Sanglah Denpasar. The study period was from Jan 2003 until Apr 2004.

Inclusion criteria were patients who had been diagnosed dyspepsia and would undergo upper gastrointestinal endoscopic examination, with age more than 15 years old. Patients were not taking antibiotics, H₂ receptor antagonists or proton pump inhibitors during 7 d prior to the examination.

Patients who had fulfilled inclusion criteria had endoscopic examination and biopsy samples were taken two in the antrum and two more in the corpus. Pronto Dry (Medical Instruments Corporation, Solothurn, Switzerland) was performed according to standard procedure and interpretation was read in 1 h after examination.

Biopsies for histopathologic examination were fixed with formalin and sent to Histopathologic Department to be analyzed and confirm the presence of *H pylori* infection. If *H pylori* was found positive, its density would be read semi quantitatively. Histopathologic examination of gastric biopsy samples was interpreted based on the Updated Sydney system classification.

Statistical analysis

The values are expressed as mean \pm SD. Statistical calculation to test sensitivity, specificity, positive predictive value, negative predictive value, and the accuracy was done using statistics software. $P < 0.05$ was taken as significant.

RESULTS

This multicentre study was conducted in 6 endoscopic centers. Each unit collected various numbers of cases and most were obtained from endoscopic room in RSCM, Jakarta. The number of patients included in the study was 550 consisting of 320 (58.2%) patients from RSCM Jakarta, 40 (7.3%) patients from RS Adam Malik Medan, 60 (10.9%) patients from RS Sutomo Surabaya, 37 (6.7%) patients from RS Sardjito Yogyakarta, 43 (7.8%) patients

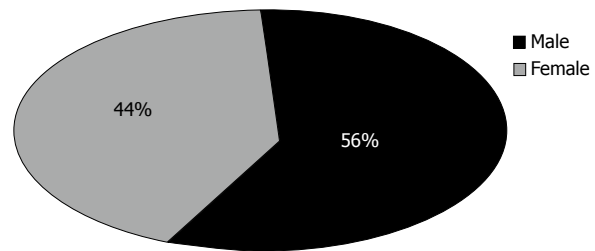


Figure 1 Distribution of patients based on gender.

Tabel 1 Results of upper gastrointestinal endoscopy of patients with dyspepsia

Endoscopic diagnosis	n	%
Normal	45	8.2
Gastritis and or duodenitis	246	44.7
Gastric ulcer	36	6.5
Duodenal ulcer	20	3.6
Mild erosive gastritis	83	15.1
Esophagitis	85	15.5
Gastric cancer	1	0.2
Mild erosive gastritis/Esohagitis	2	0.4
Severe erosive gastritis	16	2.9
Gr. B Esophagitis/Mmoderate gastritis	6	1.1
Barrett's esophagus	2	0.4
Esophagitis, gastric ulcer	1	0.2
Esophagitis, pangastritis, duodenal ulcer	1	0.2

from RS Sanglah Denpasar Bali and 50 (9.1%) patients from RS Hasan Sadikin Bandung.

Of 550 patients included in this study, 309 patients (56%) were male and 241 patients (44%) were female (Figure 1) with age ranging from 15 years to 82 years. Mean age was 44.98 ± 14.46 years. Mean age of male patients was 44.35 ± 13.85 years and of female patients was 45.78 ± 15.19 years.

Evaluation of endoscopic results showed gastric ulcer was found in 36 cases (6.5%) and duodenal ulcer in 20 cases (3.6%). Normal endoscopic finding was found in 45 cases (8.2%) and minimal disorders of gastritis and duodenitis were found in 246 cases (44.7%). In addition, one case of gastric cancer was identified (Table 1).

Diagnosis of *H pylori* infection

Based on definition determined at the beginning of the study, *H pylori* infection was considered positive if histopathologic examination showed the presence of *H pylori*. Of 550 cases studied, *H pylori* was found positive in 56 (10.2%). The highest prevalence of *H pylori* was found in patients from Yogyakarta (30.6%) and the lowest was in patients from Jakarta (8%).

Of 550 patients, 25 did not have histopathologic examinations. Furthermore, analysis on sensitivity and specificity was done with only 525 patients because they had complete examination with both histopathology and Pronto Dry. Of 56 cases which were positive based on the criteria used, 39 were positive with Pronto Dry and 17 were negative with Pronto Dry. Overall sensitivity and specificity of Pronto Dry were 69.7% and 95.7%, respectively. Positive predictive value was 66.1% and

negative predictive value was 96.4% and overall accurate rate was 92.9%. If we calculated the sensitivity and specificity of each endoscopic center, results were varied (Table 2).

DISCUSSION

To date, the rapid urease test is still needed to detect the presence of *H pylori*. This test is relatively easy and rapid and thus appropriate therapy is possible if diagnosis of *H pylori* is confirmed. In addition, upper gastrointestinal endoscopic examination is still a reliable procedure to investigate the cause of dyspepsia.

Our result indicated that endoscopic examination of patients with uninvestigated dyspepsia showed various underlying disorders. Almost 45% of endoscopic results showed organic disorders such as esophagitis, peptic ulcer and erosive gastritis. In addition, 1 of 550 cases in this study was found to be gastric carcinoma. This result indicated that endoscopy is very important in evaluating upper gastrointestinal disorder in patients with dyspepsia. Rapid urease test has become indispensable in patients having the indication for endoscopic procedure.

Of all patients with dyspepsia who had undergone endoscopic examination, *H pylori* infection was positive in only 10.2% of cases. This figure was lower than the result of multicentered study in Europe published in 1999. In that study involving 501 patients with dyspepsia, 55.7% were found to have positive result of *H pylori*. Another study conducted in Jakarta found that 9.5% patients with dyspepsia were positive for *H pylori* infection by endoscopic examination^[9].

The sensitivity, specificity, positive and negative predictive values, and overall accurate level were 69.6%, 95.7%, 66.1%, 96.4%, and 92.9%, respectively. A study conducted by Said *et al* in Malaysia to evaluate Pronto Dry found better sensitivity (98.1%) and specificity (100%)^[8]. However, if we compared the result of studies in Jakarta and Bandung, similar results to the study in Malaysia were obtained. The study in Jakarta found a sensitivity of 95.7% and specificity of 98.3%, while the study in Bandung found both sensitivity and specificity of 100%. The difference of the results might be due to different histopathologic evaluation criteria adopted by different centers. This, however, could be overcome by standardization of appropriate biopsy location, standardised type of biopsy instrument and samples size and results read by same pathologists.

Rapid urease test using Pronto Dry which is relatively new is found to have good specificity although the sensitivity is lower than specificity. Studies in several cities

Table 2 Sensitivity, specificity, positive predictive value and negative predictive value by Pronto Dry examination at each endoscopic centre

Endoscopic centre	Sensitivity (%)	Specificity (%)	Negative predictive value (%)	Positive predictive value (%)	Accurate level (%)
Jakarta	95.7	98.3	99.7	81.5	98.2
Bandung	100.0	100.0	100.0	100.0	100.0
Medan	100.0	59.4	100.0	38.1	67.5
Surabaya	33.3	98.1	93.0	66.7	91.6
Yogyakarta	10.0	100.0	75.0	100	75.7
Bali	25.0	94.1	84.2	50.0	81.0

have shown that Pronto Dry has very good sensitivity and specificity level above 95%. If we compare with the other rapid urease test, Pronto Dry can give result in only 1 h. This test seems very promising as a diagnostic tool to detect *H pylori* more rapidly and accurately.

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Metastatic breast cancer to the gastrointestinal tract: A case series and review of the literature

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Abstract

Metastatic breast cancer involving the hepatobiliary tract or ascites secondary to peritoneal carcinomatosis has been well described. Luminal gastrointestinal tract involvement is less common and recognition of the range of possible presentations is important for early and accurate diagnosis and treatment. We report 6 patients with a variety of presentations of metastatic breast cancer of the luminal gastrointestinal tract. These include oropharyngeal and esophageal involvement presenting as dysphagia with one case of pseudoachalasia, a linitis plastica-like picture with gastric narrowing and thickened folds, small bowel obstruction and multiple strictures mimicking Crohn's disease, and a colonic neoplasm presenting with obstruction. Lobular carcinoma, representing only 10% of breast cancers is more likely to metastasize to the gastrointestinal tract. These patients presented with gastrointestinal manifestations after an average of 9.5 years and as long as 20 years from initial diagnosis of breast cancer. Given the increased survival of breast cancer patients with current therapeutic regimes, more unusual presentations of metastatic disease, including involvement of the gastrointestinal tract can be anticipated.

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Key words: Breast carcinoma; Metastasis; Gastrointestinal tract

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INTRODUCTION

Breast cancer is the most common neoplasm in women, accounting for approximately 32% of cancers in women, with a lifetime risk of 1 in 10^[1]. Prognosis is related to the presence of hormonal receptors, the size and grade of the primary tumour, the presence of regional lymphadenopathy, and metastatic disease^[1]. Metastatic breast cancer typically involves the lungs, bones, brain and liver, but occasionally can affect the gastrointestinal tract^[2]. We report 6 patients with a variety of presentations involving the gastrointestinal tract, all of which were eventually found to be due to metastatic breast cancer. These examples highlight the importance of considering metastatic breast cancer as a potential cause of radiological abnormalities affecting any part of the gastrointestinal tract of women, especially those with a previous history of breast cancer.

CASE REPORTS

Case 1

A 51-year old female presented with a 3-mo history of progressive solid food dysphagia. Initial barium swallow and endoscopy were normal. Esophageal manometry showed impaired relaxation of the lower esophageal sphincter, but no other abnormality. A decision was made to follow the patient and re-investigate her in 6 mo. The patient returned after 5 mo with worsening dysphagia and 20-pound weight loss. Barium swallow demonstrated retained secretions, esophageal dilatation and tapered narrowing in the region of the lower esophageal sphincter (Figure 1). Repeat manometry demonstrated changes consistent with achalasia. Repeat endoscopy showed a dilated esophagus and a narrowed gastroesophageal junction with no intrinsic mucosal abnormality. Pneumatic dilatation with a Rigiflex 35 mm balloon was unsuccessful and the patient was referred for thoracoscopic myotomy. At the time of surgery, the left lower lobe of the lung was found to be fixed to the gastroesophageal junction by tumor that extended to involve the distal esophagus and pericardium. Biopsies showed infiltrating lobular breast carcinoma. The patient had a right mastectomy 15 years previously. The patient recovered from surgery and was treated with tamoxifen with gradual normalization of her swallowing function. She declined repeat manometric or radiological assessment.

Case 2

A 58-year old female with a previous history of Paget's disease



Figure 1 Barium swallow demonstrating smooth, symmetric tapering of the esophagus (arrow), suggesting achalasia.

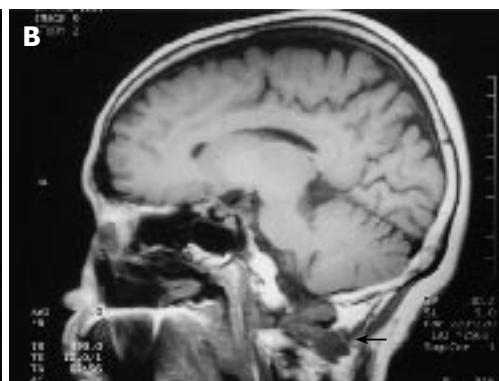


Figure 2 Sagittal T1-weighted MRI demonstrating low-signal consistent with marrow replacement at both C1 and the posterior vertebral body of C3 (arrow) (A), and low-signal soft tissue mass adjacent to the C2 vertebral body (arrow) (B).



Figure 3 Barium swallow demonstrating irregular stenosis (arrow) involving the middle esophagus.

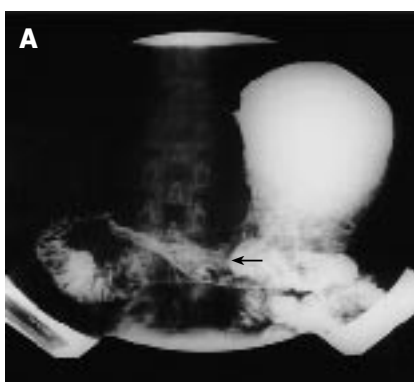


Figure 4 Double-contrast upper GI examination demonstrating irregular narrowing of the gastric antrum (arrow) (A), CT demonstrating gastric mural thickening, perigastric stranding, (arrow) and lymphadenopathy (B).

of the bone involving the cervical spine, multinodular goitre and neck trauma secondary to a motor vehicle accident presented with oropharyngeal dysphagia and mild dysarthria. Barium swallow demonstrated poor bolus formation, decreased laryngeal elevation, incomplete oropharyngeal clearance, and aspiration of barium. Electromyography was suspicious of a motor neuron disease, demonstrating paralysis of the left vocal cord, tongue fasciculations, and denervation of the left sternocleidomastoid. The patient underwent magnetic resonance imaging (MRI) which demonstrated a soft tissue density at the C2 level, as well as marrow replacement in several vertebrae (Figure 2A and 2B). Open biopsy of the soft tissue showed adenocarcinoma consistent with metastatic breast cancer. The patient had a modified left radical mastectomy for ductal adenocarcinoma followed by radiation therapy 20 years previously.

Case 3

A 66-year old female presented with solid food dysphagia for several months. Barium swallow demonstrated a mid-esophageal stricture that was suspicious of neoplastic disease (Figure 3). Endoscopy and biopsy confirmed metastatic infiltration of the esophagus by lobular breast carcinoma. Unfortunately, attempted palliative dilatation resulted in perforation of the esophagus. The subsequent

course of events resulted in prolonged hospitalization with multiple surgical procedures, complications and eventually death. The patient had a modified right mastectomy and radiotherapy 13 years previously for node negative malignancy. The patient had known metastatic disease to the left hilum, proven by biopsy, 6 years prior to the presentation of dysphagia.

Case 4

An 80-year old female presented with a history of early satiety and vomiting. Double contrast upper gastrointestinal barium examination was consistent with linitis plastica (Figure 4A). Endoscopy with biopsy of the gastric body and antrum confirmed metastatic infiltrating lobular carcinoma of breast origin. Subsequent computed tomography (CT) scanning demonstrated changes in the stomach and duodenum (Figure 4B). The patient received palliative care, but her symptoms were progressed after 14 mo. Plain films of the abdomen demonstrated a small bowel obstruction, suspected to be due to intra-abdominal spread of the disease. The patient underwent modified left radical mastectomy 2 years previously and had node positive disease.

Case 5

A 60-year old female presented with a history of diarrhea,

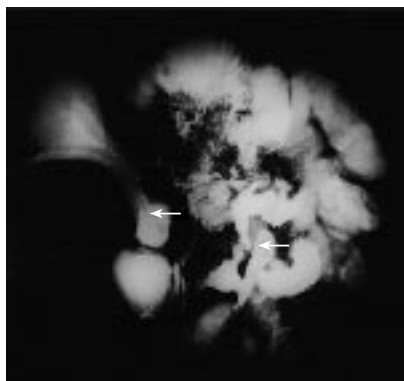


Figure 5 Small bowel enteroclysis showing persistent narrowing of the gastric body and antrum (arrow), prominent irregular gastric rugae with diffuse mucosal serration, and strictures of the ileum.

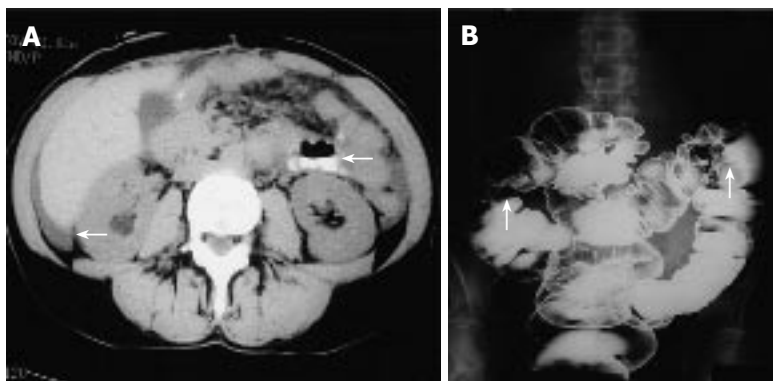


Figure 6 CT demonstrating a dilated loop of small bowel (arrow) and ascites (A), double-contrast barium enema demonstrating fixed eccentric strictures of the ascending colon and splenic flexure (arrows) with a third obscuring lesion seen within the sigmoid colon but not projected in profile on this image (B).

abdominal cramps and vomiting. Crohn's disease was suspected and she was initially treated with prednisone with clinical improvement. Several months later she experienced an exacerbation of her symptoms and presented with a partial small bowel obstruction. Small bowel enteroclysis demonstrated multiple abnormalities, including persistent narrowing of the gastric body and antrum (Figure 5), prominent irregular gastric rugae with diffuse mucosal serration, as well as 3 separate strictures of the ileum. The findings were compatible with the diagnosis of Crohn's disease, but upper gastrointestinal endoscopy showed gross findings suspicious of malignancy. Biopsy demonstrated adenocarcinoma of breast origin. CT examination demonstrated ascites and mesenteric involvement, subsequently confirmed at laparotomy. The patient had a modified radical mastectomy 10 years previously.

Case 6

A 51-year old female had a 3-year history of increasing abdominal pain. CT examination demonstrated a dilated small bowel as well as ascites (Figure 6A). Subsequent laparotomy demonstrated that the patient had a small bowel obstruction secondary to carcinomatosis consistent with metastatic breast cancer. After surgical treatment and hormonal therapy, the patient improved clinically. One year later, she presented with diarrhea. A double contrast barium enema demonstrated lesions in the ascending, distal transverse and sigmoid regions of the colon (Figure 6B). The appearance of the lesions was consistent with synchronous colonic cancers, but proved to be metastatic breast adenocarcinoma on pathology. The patient was diagnosed with breast cancer 4 years prior to the presentation with small bowel obstruction.

DISCUSSION

Breast cancer accounts for 19% of cancer deaths in women^[1]. Current therapies have had a significant impact on the mortality and survival rates of breast cancer^[3]. Prognosis is related to several factors including the presence of metastases with tumor histology being one of

the predictors of metastatic spread. For example, tubular and mucinous carcinomas have a lower incidence of metastases and a better prognosis^[4]. Lobular carcinoma, though less common and by mechanisms that are not clear, is more likely to metastasize to the gastrointestinal tract^[4]. Although the gastrointestinal tract is a less common site for metastatic involvement by breast cancer, recognising the range of possible presentations is important for early and accurate diagnosis and treatment. The cases we described in this report highlight these features. Also, our patients presented with gastrointestinal manifestations after an average of 9.5 years and as long as 20 years of initial diagnosis. A 30-year interval between the original diagnosis and the presentation of metastatic disease has been reported in the literature^[5]. Given the increased survival of breast cancer patients with current therapeutic regimes, more unusual presentations of metastatic disease, including involvement of the gastrointestinal tract can be anticipated^[2,6,7], and hence this diagnosis should be considered in any woman with a previous history of the disease presenting with new gastrointestinal complaints.

Previous case reports and small series that have documented gastrointestinal involvement by metastatic breast cancer, together with the cases presented here, are summarized in Table 1. Any region of the gastrointestinal tract can be involved. However, oropharyngeal and esophageal involvement presenting as dysphagia is unusual^[8]. Our series is unique in that it includes three examples of this: case 1 with pseudoachalasia, case 2 with oropharyngeal dysphagia secondary to nerve involvement, and case 3 with malignant esophageal stricture. In our review of the literature, this is only the third reported case of pseudoachalasia secondary to metastatic breast cancer^[9,10]. As well, this is the first reported case of oropharyngeal dysphagia resembling case 2. There is a previous report of one case of metastases to the tongue^[11]. Esophageal involvement most commonly presents as a short-segment of extrinsic obstruction in the mid-esophagus and has been reported to occur up to 22 years following the initial diagnosis of breast cancer^[8]. Mediastinal and hilar lymphadenopathy may be present^[12], and endoscopic biopsy may not provide the diagnosis

if the obstruction is extrinsic and does not involve the mucosa. Long segment involvement of the esophagus, up to 20 cm, has been described^[13]. Symptoms as well as radiographic findings may mimic radiation-induced or peptic stricture^[12].

The stomach is the most common site of gastrointestinal involvement by metastatic breast cancer with an incidence of up to 15% at autopsy^[12,14]. The usual presentation mimics linitis plastica and is typically due to lobular carcinoma^[6], as illustrated by case 4. Findings include gastric wall thickening, rigidity and decreased peristalsis. Cases in which the linitis plastica-like presentation precedes the diagnosis of breast cancer have been documented^[6]. In one series of 22 patients, endoscopy and biopsy could provide the correct diagnosis in only 13, most likely because tumor infiltration is deep to the mucosa^[15]. Metastatic involvement of the stomach can mimic primary gastric neoplasms and inflammatory conditions such as peptic ulcer or gastritis^[16]. Bleeding and perforation have also been reported^[17-19].

Malignant neoplasms of the small intestine are uncommon and are usually primary adenocarcinomas^[20]. Metastatic involvement of the small bowel can be due to malignant melanoma, or cancer of the colon, cervix, ovary or rarely esophagus^[12,20]. Metastatic involvement of the small intestine is recognized at autopsy^[21,22], but clinical presentation is uncommon. Patients can present with abdominal pain and diarrhea^[20], similar to case 5, intussusception^[12] or even appendicitis^[7]. The terminal ileum is involved more often than the proximal small bowel, possibly due to the tracking of peritoneal fluid along the mesentery into the right lower quadrant. The clinical and radiological presentation can mimic Crohn's disease, as illustrated by case 5. Metastatic disease to the duodenum has also been described^[23].

Colonic involvement is common with some series showing colonic metastases in up to 12% of patients with breast cancer^[22,24]. Carcinoma of the breast is the most frequent source of hematogenous metastases to the large bowel^[2], but involvement via peritoneal and lymphatic spread also occurs^[12]. As in the stomach, the lobular form of breast cancer is more likely to metastasise to the colon^[2,4]. The presentation can be similar to primary neoplasia of the colon or can mimic Crohn's disease, both clinically and radiologically^[2,12,16,15]. Air contrast barium enema or CT may demonstrate diffuse or multifocal involvement^[16]. Mucosal nodularity, stenosis, decreased distensibility, angulation and tethering are also characteristic findings^[12]. Solitary apple-core lesions are less common^[15]. Similar to the upper gastrointestinal tract, endoscopy and biopsy provide only a moderate diagnostic yield: 6 of 10 patients in one series^[15]. Deep biopsy or laparotomy may be required for diagnosis^[18,15]. Rectal involvement has also been described, including rectal stenosis secondary to metastatic breast cancer^[25,26].

Other intra-abdominal sites of metastatic breast cancer that do not involve the gastrointestinal tract per se have been reported. Ascites, secondary to peritoneal carcinomatosis, is a common abnormality demonstrated by abdominal CT^[27]. Metastatic spread to the hepatobiliary tract is well described^[19,28]. Metastases to the diaphragm,

Table 1 Summary of gastrointestinal presentations of metastatic breast cancer

Site	Presentation	Reference
Oropharynx	Oropharyngeal dysphagia Tongue lesion	(Case 2) 11
Esophagus	Stricture Achalasia Non-specific dysmotility	(Case 3), 8, 13, 31-44 (Case 1), 9, 10 30
Stomach	Linitis plastica Obstruction/Stenosis Polyp Ulcer/Erosion Perforation	(Case 4), 5, 6, 45-52 (Case 5), 19, 45 45, 53, 54 19, 45, 55, 56 57
Small intestine	Obstruction/Stenosis Multiple strictures/ Diffuse infiltr	(Case 4), (Case 6), 20, 58-61 (Case 5)
Colon	Obstruction/Stenosis Asymptomatic abdominal Mass Multiple strictures/ Diffuse infiltr Polyp	25, 59, 62-65 19 (Case 6), 66 67
Rectum	Obstruction/Stenosis Linitis plastica	15, 25, 26, 68 47

the genitourinary tract, the retroperitoneum, the mesenteric lymph nodes, the abdominal wall and subcutaneous tissues have been reported^[11,12,27]. Spread to the ovaries is identified histologically, but is not usually clinically evident or demonstrated with CT in the absence of other evidence for metastatic disease^[29].

Metastatic breast cancer involving the gastrointestinal tract can produce a wide range of clinical and radiological presentations, often mimicking other gastrointestinal disorders. Given the high prevalence of this disease, breast cancer needs to be considered in any women presenting with new gastrointestinal complaints, especially those with a history of breast cancer, even if the initial diagnosis was made many years previously.

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Pancreatic cancer with a high serum IgG4 concentration

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Abstract

Differentiation between autoimmune pancreatitis and pancreatic cancer is sometimes difficult. It has been reported that serum IgG4 concentrations are significantly elevated and particularly high (>135 mg/dL) in autoimmune pancreatitis. Measurement of serum IgG4 has become a useful tool for differentiating between autoimmune pancreatitis and pancreatic cancer. However, we present a 74-year-old female with a markedly elevated serum IgG4 (433 mg/dL) who underwent pancreaticoduodenectomy for pancreatic cancer. Elevated serum IgG4 levels continued after the resection. On histology, adenocarcinoma of the pancreas accompanied with moderate lymphoplasmacytic infiltration infiltrated the lower bile duct and duodenum, but there were no findings of autoimmune pancreatitis. Although a small metastasis was detected in one parapancreatic lymph node, regional lymph nodes were swollen. Abundant IgG4-positive plasma cells infiltrated the cancerous areas of the pancreas, but only a few IgG4-positive plasma cells were detected in the noncancerous areas. Pancreatic cancer cells were not immunoreactive for IgG4. An abundant infiltration of IgG4-positive plasma cells was detected in the swollen regional lymph nodes and in the duodenal mucosa. We believe that the serum IgG4 level was elevated in this patient with pancreatic cancer as the result of an IgG4-related systemic disease that had no clinical manifestations other than lymphadenopathy.

Kamisawa T, Chen PY, Tu Y, Nakajima H, Egawa N, Tsuruta K, Okamoto A, Hishima T. Pancreatic cancer with a high serum IgG4 concentration. *World J Gastroenterol* 2006; 12(38): 6225-6228

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INTRODUCTION

Autoimmune pancreatitis is a unique form of pancreatitis, histopathologically characterized by dense lymphoplasmacytic infiltration and fibrosis of the pancreas with obliterative phlebitis^[1,2]. Patients with this disease usually have a swollen pancreas with delayed enhancement on computed tomography (CT), irregular narrowing of the main pancreatic duct on endoscopic retrograde pancreatography, and a favorable response to steroid therapy^[1-4]. Autoimmune pancreatitis has been frequently mistaken for pancreatic cancer for 3 major reasons: most patients are elderly (average age of 59^[4]-61^[5] years); painless obstructive jaundice is present due to the associated sclerosing cholangitis (65%^[4]-86%^[6]); and there is a mass in the pancreas.

In 2001, Hamano *et al*^[7] reported that serum IgG4 concentrations were significantly elevated and particularly high in autoimmune pancreatitis and were closely associated with disease activity. They measured serum IgG4 levels in 20 patients with autoimmune pancreatitis and 70 patients with pancreatic cancer. A cutoff value for the serum IgG4 level of 135 mg per deciliter resulted in a high rate of accuracy (97%), sensitivity (95%), and specificity (97%) for the differentiation of autoimmune pancreatitis from pancreatic cancer. Since this report, IgG4 has become a serological marker of autoimmune pancreatitis^[8,9]. Hirano *et al*^[10] also stated that serum IgG4 concentrations are helpful for differentiating between autoimmune pancreatitis and pancreatic cancer, as none of their 23 patients with pancreatic cancer had a high serum IgG4 level. Here, we present a resected case of pancreatic cancer that had a high serum IgG4 level and discuss the possible mechanisms for the elevated serum IgG4 levels in this patient.

CASE REPORT

A 74-year-old female was admitted to our hospital in May 2005 complaining of jaundice. She had no history of

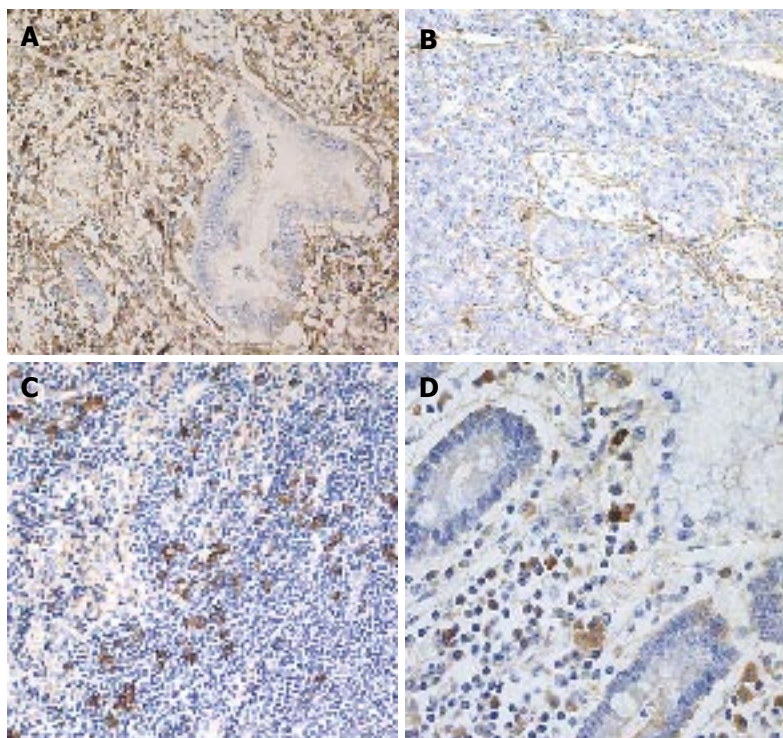


Figure 1 Immunostaining of IgG4 in the resected specimens. Abundant IgG4-positive plasma cells were detected in the cancerous lesion of the pancreas (A) but only a few IgG4-positive plasma cells were seen in the noncancerous areas of the pancreas (B). Infiltration of abundant IgG4-positive plasma cells was detected in regional lymph node (C) and the duodenal mucosa (D).

other illness or alcohol abuse. On admission, there was no swelling of the salivary glands and the cervical lymph nodes were not palpable. Laboratory data included: total bilirubin 7.4 mg/dL (normal: 0.2-1.1 mg/dL), alkaline phosphatase 1466 IU/L (115-359 IU/L), aspartate aminotransferase 236 IU/L (11-25 IU/L), and alanine aminotransferase 431 IU/L (4-27 IU/L). Serum levels of tumor markers DUPAN 2 and Span 1 were elevated to 7526 U/mL (< 150 U/mL) and 101 U/mL (< 30 U/mL), respectively.

Abdominal ultrasonography demonstrated a 4 cm-sized hypoechoic mass in the pancreatic head with dilatation of the intrahepatic and extrahepatic bile ducts and the main pancreatic duct. On helical CT scan, the mass was hypoattenuated to the pancreas in the early phase, but attenuation increased in the delayed phase. Based on the CT finding of delayed enhancement, we examined the serological findings, suspecting autoimmune pancreatitis. Although the serum IgG was 1560 mg/dL (870-1700 mg/dL) and the antinuclear antibody test (ANA) was negative, the serum IgG4 measured by single radial immunodiffusion (The Binding Site, Birmingham, UK) was elevated to 433 mg/dL.

Endoscopic retrograde cholangiopancreatography showed stenosis of the lower portion of the common bile duct and the main pancreatic duct near the major duodenal papilla, and endoscopic nasobiliary drainage was performed. Histological examination of the biopsy specimen from the irregular erosive duodenal mucosa around the major duodenal papilla under duodenoscopy revealed infiltration of adenocarcinoma.

Given the diagnosis of pancreatic head cancer, a pancreaticoduodenectomy was performed. Histological examination revealed that moderately differentiated tubular adenocarcinoma arising in the head of the pancreas

infiltrated to the lower bile duct and duodenum. A small histological metastasis was detected in one parapancreatic lymph node. Although moderate lymphoplasmacytic infiltration was observed in the cancerous areas, no findings of autoimmune pancreatitis were detected in the noncancerous areas of the pancreas.

The specimens resected during pancreaticoduodenectomy were fixed in 10% formaldehyde. Serial sections (4 μ m thick) were cut from paraffin embedded tissue blocks. Sections of the pancreas, including the extrahepatic bile duct, gallbladder, duodenum, stomach and regional lymph nodes were immunostained using anti-CD4-T (Novocastra, Newcastle upon Tyne, UK), CD8-T (DakoCytomation, Glostrup, Denmark), and IgG4 (The Binding Site) with avidin-biotin-peroxidase complex (ABC). The number of immunohistochemically identified IgG4 positive plasma cells was counted per high power field (hpf). Four fields were analyzed per section. Immunohistochemically, abundant IgG4-positive plasma cells (> 20/hpf) and CD4- or CD8-positive T lymphocytes were seen infiltrating the cancerous lesion of the pancreas (Figure 1A), but only a few IgG4-positive plasma cells (< 3/hpf) were detected in the noncancerous areas of the pancreas (Figure 1B). Pancreatic cancer cells were not immunoreactive for IgG4. Regional lymph nodes were swollen up to 1.5 cm in diameter, and infiltration of abundant IgG4-positive plasma cells (> 20/hpf) was detected in almost all regional lymph nodes (Figure 1C). Abundant IgG4-positive plasma cells infiltrated the duodenum (Figure 1D), but there were only a few IgG4-positive plasma cells (< 3/hpf) detected in the extrahepatic bile duct, gallbladder, and gastric mucosa.

The patient was given chemotherapy as an outpatient and was well 6 mo later. Since the operation, her serum IgG4 levels have continued to be elevated (Figure 2).

DISCUSSION

Clinically, patients with autoimmune pancreatitis and pancreatic cancer share many features, including being elderly, having painless jaundice, new-onset diabetes mellitus, and elevation of tumor markers^[1-4]. Radiologically, focal swelling of the pancreas, the “double-duct sign” representing stricture in both the biliary and pancreatic ducts, and angiographic abnormalities seen in pancreatic cancer patients are sometimes also observed in patients with autoimmune pancreatitis^[1-4]. As autoimmune pancreatitis responds dramatically to steroid therapy^[1-4,11], an accurate diagnosis of autoimmune pancreatitis can avoid unnecessary laparotomy or pancreatic resection. Imaging findings, such as a mass showing delayed enhancement and a capsule-like rim on dynamic CT, and a narrowing of the pancreatic duct associated with a less dilated upstream pancreatic duct, are all useful in differentiating pancreatic cancer from autoimmune pancreatitis^[3,4]. Histopathological approach on biopsy is necessary in some cases, but it is sometimes difficult to obtain sufficient pancreatic tissue biopsy. Since Hamano *et al.*^[7] reported that serum IgG4 concentrations were significantly elevated and particularly high in autoimmune pancreatitis, measurement of serum IgG4 has become a useful tool in the differentiation of autoimmune pancreatitis and pancreatic cancer^[8-10].

However, the present case had a histologically proven pancreatic cancer and displayed marked elevation of serum IgG4 concentration. The patient did not have any pathological conditions such as atopic dermatitis, parasitic disease, pemphigus vulgaris and foliaceus, which are sometimes associated with elevated serum IgG4 levels^[7,9]. Interestingly, elevated serum IgG4 levels continued after the resection of the pancreatic cancer. Characteristic histological feature of autoimmune pancreas include dense lymphoplasmacytic infiltration and fibrosis of the pancreas^[1,2]. In our patient, moderate lymphoplasmacytic infiltration was observed in the cancerous lesion, but an inflammatory process was not detected in the noncancerous areas of the pancreas. That is, there were no histological findings that suggested autoimmune pancreatitis in this patient's pancreas. Immunohistochemically, pancreatic cancer cells were not positive for IgG4. Abundant IgG4-positive plasma cells infiltrated the cancerous areas of the pancreas, but only a few IgG4-positive plasma cells were detected in the noncancerous areas of the pancreas. Furthermore, many IgG4-positive plasma cells infiltrated almost all of the swollen regional lymph nodes and the duodenum.

Autoimmune pancreatitis is sometimes associated with sclerosing cholangitis, sclerosing sialadenitis, retroperitoneal fibrosis, and lymphadenopathy^[12,13]. During follow-up of sclerosing sialadenitis, some patients developed autoimmune pancreatitis^[13]. We have reported that lymphoplasmacytic infiltration with fibrosis was observed in the peripancreatic retroperitoneal tissue, biliary tract, and salivary glands, as well as in the pancreas of patients with autoimmune pancreatitis^[13]. Furthermore, an abundant infiltration (> 30/hpf) of IgG4-positive plasma cells together with CD4- or CD8-positive T lymphocytes was observed in the various organs of

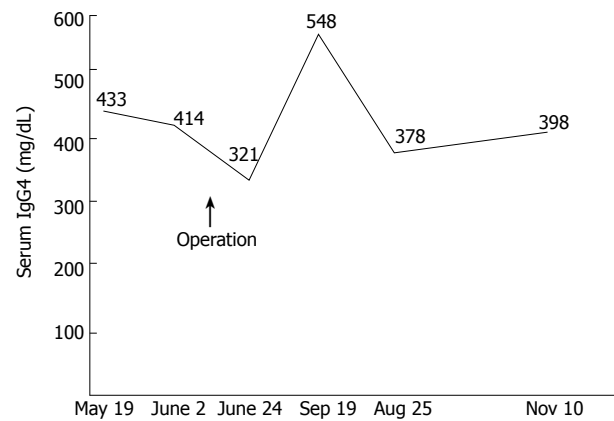


Figure 2 Course of serum IgG4 levels in this patient. Elevated serum IgG4 levels continued after operation.

patients with autoimmune pancreatitis, including the peripancreatic retroperitoneal tissue, biliary tract, salivary glands, gastric mucosa, lymph nodes, and pancreas, but an abundant infiltration of IgG4-positive plasma cells was not detected in the organs of patients with chronic alcoholic pancreatitis^[13-15]. We therefore proposed the existence of a novel clinicopathological entity, an IgG4-related systemic disease incorporating sclerosing pancreatitis, cholangitis, sialadenitis, and retroperitoneal fibrosis with lymphadenopathy, characterized by extensive IgG4-positive plasma cell and CD4- or CD8-positive T lymphocyte infiltration of organs^[14,15]. We also suggested that autoimmune pancreatitis is not simply a pancreatitis but that, in fact, it is a pancreatic lesion reflecting an IgG4-related systemic disease, and that in some cases only organs other than the pancreas might be clinically involved^[14,15].

In our patient, the IgG4-positive plasma cells infiltrating the lymph nodes were as abundant as those in the lymph nodes of patients with autoimmune pancreatitis, and elevation of serum IgG4 levels continued after the resection. We believe that the serum IgG4 level was elevated in this patient with pancreatic cancer as the result of an IgG4-related systemic disease that had no clinical manifestations other than lymphadenopathy. Furthermore, this case calls into question the specificity of elevated IgG4 levels for benign disease, and suggests that serum IgG4 levels should be interpreted with caution in patients with a mass in the pancreas but no histological documentation of either carcinoma or pancreatitis.

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Biliary stone causing afferent loop syndrome and pancreatitis

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Abstract

We report the case of an 84-year-old female who had a partial gastrectomy with Billroth-II anastomosis 24 years ago for a benign peptic ulcer who now presented an acute pancreatitis secondary to an afferent loop syndrome. The syndrome was caused by a gallstone that migrated through a cholecystoenteric fistula. This is the first description in the literature of a biliary stone causing afferent loop syndrome.

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Key words: Afferent loop syndrome; Biliary stone; Acute pancreatitis; Gallstone ileus

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INTRODUCTION

The afferent loop syndrome is a mechanical obstruction that impairs the clearance of bile and pancreatic juices from the afferent jejunal loop of a gastrojejunostomy. Entrapment of the afferent loop by postoperative adhesions, internal hernias, loop kinking at the gastrojejunostomy, intestinal volvulus, intussusception, anastomotic cancer, enteroliths and bezoars have all been incriminated as causes. A cholecystoenteric fistula with biliary stone impaction in the anastomosis as a cause has never been reported, as far as we know.

CASE REPORT

An 84-year-old female presented in the emergency room with severe right upper quadrant pain and vomiting in the last 8 h. She referred cholecystolithiasis with infrequent episodes of pain; the last one extended for 6 d and ended

4 d ago, being longer and more severe than usual. She brought a previous abdominal sonography showing a single stone of 1.5 cm in the gallbladder.

Her past history included diabetes mellitus and a partial gastrectomy with a Billroth II reconstruction for benign peptic ulcer 24 years ago. On physical examination the patient reported intense pain in the superior half of the abdomen. Vital signs were stable. Laboratory exams showed mild leukocytosis and acute pancreatitis.

An abdominal sonography was performed and demonstrated air inside the gallbladder associated with a dilatation of the afferent jejunal loop, suggesting a gallstone ileus as a possible cause for the bowel distension.

A computed tomography was then performed and revealed an afferent loop syndrome due to a stone impacted in the gastrojejunal anastomosis. Pancreatitis was also observed (Figure 1, Figure 2, Figure 3, Figure 4).

A few hours after the admission the patient presented hypotension and a decline in her mental status requiring orotracheal intubation and vasopressors. She was then transferred to an intensive care unit.

An upper digestive endoscopy was performed and successfully removed the stone with a basket (Figure 5). During the following two days her white blood cell count increased, her renal and liver functions declined and the vasopressors were progressively augmented. She also presented an abdominal compartment syndrome on the second day being submitted to a decompressive laparotomy. During the laparotomy adhesions were observed between the afferent loop and the gallbladder.

On the third day the patient presented a cardiac arrest and died.

DISCUSSION

The afferent loop syndrome results from the obstruction of the afferent jejunal loop of a gastrojejunostomy. Its acute form usually occurs in the early postoperative period but it can occur decades after the surgery having a devastating course unless promptly treated^[1-4].

There are various causes of the afferent loop syndrome. Adhesions, volvulus, intussusception are some of the most common while enteroliths, cancer and bezoars are some of the rarest^[1-7]. This is the first report in the literature of a biliary stone causing the syndrome (Pubmed, Medscape, Cochrane database and Scielo database were systematically reviewed).

The pain reported by the patient 4 d before the admission might have been an acute cholecystitis that led to the formation of a cholecystoenteric fistula, allowing the biliary stone to ingress the afferent loop and ultimately

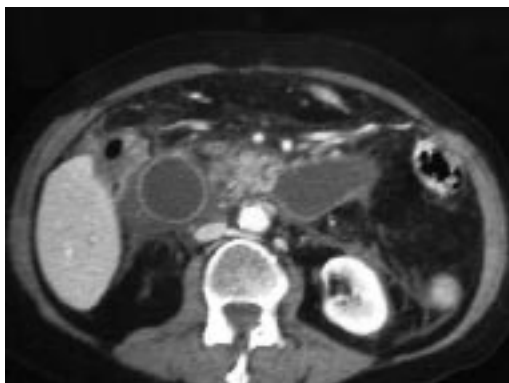


Figure 1 Dilated jejunal loop crossing the midline.

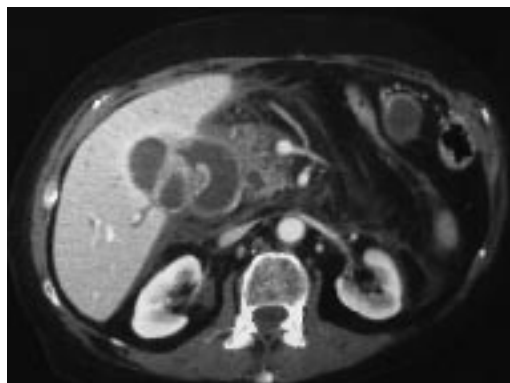


Figure 2 The dilated afferent loop and the stone (arrow).



Figure 3 Stone in more detail (arrow).



Figure 4 The relation between the gastrojejunal anastomosis (right arrow) and the stone (left arrow) in a tomographic reconstruction.

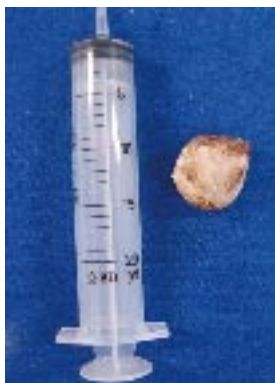


Figure 5 Biliary stone (with a diameter of 3 centimeters) after endoscopic removal.

stop in the anastomosis causing the obstruction.

Concerning the acute pancreatitis it is an uncommon and aggressive manifestation of the afferent loop syndrome with few cases found in the literature. The transmission of high pressures back to the biliopancreatic ductal system leading to duodenopancreatic reflux seems to play an important role in its pathogenesis. An experimental model of “closed duodenal loop” can simulate this clinical situation^[4,6,8].

Regarding the management of the obstruction, the choice to perform a gastrointestinal endoscopy was based on the patient's vital signs that were quickly declining. In fact this is a rapid procedure, with low risk of complications, far less aggressive than a laparotomy and it has already been successfully used to remove gallstones

occluding the upper digestive tract^[9,10]. Percutaneous drainage is also a conservative option^[11]. Either way surgical lithotomy can always be performed, even as a last resource when the other options failed^[12].

Although the obstruction was rapidly resolved and the measures for severe pancreatitis were quickly introduced the multiple organ dysfunction was already installed. The patient's age and the diabetes also contributed to the outcome.

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CASE REPORT

Propylthiouracyl-induced severe liver toxicity: An indication for alanine aminotransferase monitoring?

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Abstract

Propylthiouracyl (PTU)-related liver toxicity is likely to occur in about 1% of treated patients. In case of acute or subacute hepatitis, liver failure may occur in about one third. We report two further cases of PTU-induced subacute hepatitis, in whom the delay between occurrence of liver damage after the initiation of treatment, the underestimation of its severity and the delayed withdrawal of the drug were all likely responsible for liver failure. The high incidence of liver toxicity related to PTU, its potential severity and delayed occurrence after initiation of treatment are in favor of monthly alanine aminotransferase monitoring, at least during the first six months of therapy.

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Key words: Hepatitis; Drug-induced liver toxicity; Propylthiouracyl; Alanine aminotransferase

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INTRODUCTION

Propylthiouracyl (PTU)-related severe hepatitis remains a relatively rare occurrence^[1,2] even if a transient increase in liver function tests is observed following the initiation of treatment in 15% to 28% of cases^[2-4]. Before 1998, only 25 cases of PTU-induced severe liver injury were reported^[5] and 83 cases with 28 deaths were included in a recent review of literature^[6]. We here report two additional cases of PTU-induced severe liver injury, one was complicated

by liver failure with hepatic encephalopathy. The late occurrence of liver toxicity and the underestimation of liver damage together with delayed drug withdrawal likely play an important role in their severity.

CASE REPORT

Case 1

A 35-year old woman was referred to our unit on February 4, 2004 for a two-month history of jaundice and pruritus. She was treated for hyperthyroidism with thiamazol, thiamazol and levothyroxin in 1997-1999. In early July 2003 three months following an uneventful delivery, she presented with features of relapsing hyperthyroidism, with her thyroid-stimulating hormone (TSH) unmeasurable (normal range: 0.2-3.5 microU/mL), T4 > 6 ng/dL (normal range: 0.8-2 ng/mL) and a strong positivity of antithyroid antibodies. She was treated with PTU (150 mg per day during the first 2 mo followed by 100 mg per day). In November 2003, she experienced upper abdominal discomfort, nausea and vomiting together with a weight-loss of 4 Kg. Thyroid testing performed on December 16 showed: TSH: 0.04, T4: 0.95, free T3: 9.71. Liver biochemistry showed an increase in aspartate aminotransferase (AST) at 495 IU/L (normal value < 33) and ALT at 1575 IU/mL (normal value < 63). The total serum bilirubin level was 10 mg/dL. Despite this worsening in liver function test (LFT), therapy with PTU was however continued till its delayed withdrawal on January 21, 2004. She was referred to our unit on February 4, 2004 due to jaundice and fatigue together with the persistence of upper gastrointestinal (GI) symptoms.

She was deeply icteric with a hepatomegaly 4 cm under the right costal margin. Liver biochemistry showed alanine aminotransferase (ALT): 458 IU/L, AST: 307 IU/L, alkaline phosphatase (Alk. Phos): 139 IU/L (normal value < 94 IU/L), lactate dehydrogenase (LDH): 239 IU/L (normal value < 192 IU/L), and total s. bilirubin at 22.2 mg/dL. Serology for hepatitis A, B, C, CMV, EBV and herpes was negative. There was a slight positivity of pANCA and cANCA (1/160). At the time of admission, TSH was < 0.01, T4: 3.5 and anti-TPO antibodies: 665 (normal value < 100 U/mL). Upper abdominal ultrasound was unremarkable. Magnetic resonance imaging showed the presence of bands of necrosis scattered throughout the liver parenchyma. At transjugular hepatic vein catheterisation, the hepatic venous pressure was 9 mmHg suggestive of post-sinusoidal portal hypertension. A transvenous liver biopsy specimen showed replacement

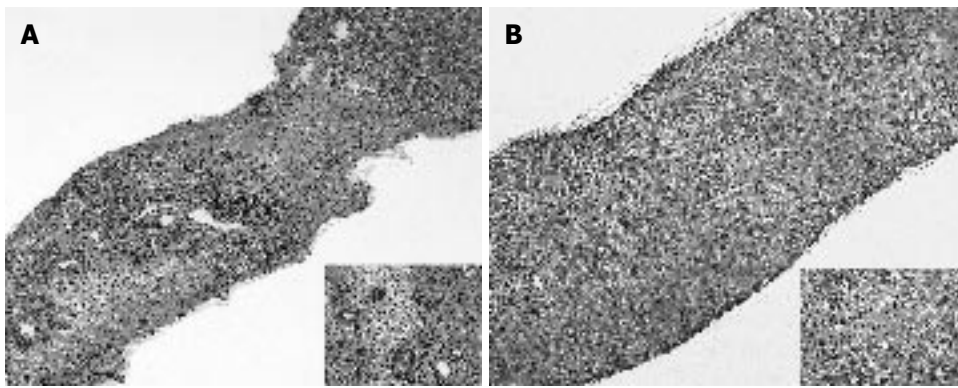


Figure 1 Liver histology of case 1 showing a picture of panlobular necrosis infiltrated with dense inflammatory infiltrates and ductular proliferation at higher magnification (close-up) (A), liver histology of case 2 showing enlarged and infiltrated portal tracts with mild interface hepatitis with prominent lobular necrosis in the centrilobular regions (close-up) (B).

of the normal architecture by areas of multilobular necrosis infiltrated by mononuclear inflammatory cells and regenerating bile ducts (Figure 1A). The few preserved portal structures were enlarged and infiltrated, also showing interface hepatitis. Shortly after admission the patient exhibited features of hepatic encephalopathy with confusion, tremor and agitation which lasted for 8 d. EEG showed features compatible with severe liver encephalopathy (grade 3 of Child's EEG scoring system). The biochemical condition then slowly improved, hyperthyroidism was treated with thiamazol (20 mg per day followed by 10 mg per day). The patient was discharged after 13 d, while her clinical and biochemical resolution occurred within 4 mo.

Case 2

A 43-year old female was referred to our unit on March 6, 2004 for a history of 10-d jaundice, fever and upper GI symptoms. She had no previous medical history except for hyperthyroidism diagnosed in August 2003. The condition was first treated with thiamazol which was however rapidly withdrawn due to the occurrence of skin rash. A treatment with PTU (100 mg per day) was initiated in early October 2003, levothyroxin (75 mg/d) was added shortly thereafter. At clinical examination upon referral, the patient was icteric with no sign of hyperthyroidism. The liver was felt 3 cm under the right costal margin. Liver biochemistry showed: total s. bilirubin: 10.9 mg/dL, AST: 2310 IU/L, ALT: 5040 IU/L. Serology was negative for hepatitis A and B as well as for antinuclear and smooth muscle antibodies. CMV-IgM as well as EBV-IgM antibodies were slightly positive. TSH was 4.09 micro U/mL, T4: 1.2, free T3: 1.9. Anti-TPO and anti-TG antibodies were both negative. Upper abdominal ultrasound showed a normal size hyperechogenic liver parenchyma. A transcutaneous liver biopsy was obtained which showed widely enlarged portal tracts infiltrated with lymphocytes and neutrophils together with ductular proliferation. Interface hepatitis was clearly visible and there were prominent areas of centrilobular necrosis infiltrated with lymphocytes in the lobules (Figure 1B). Acidophilic bodies were scattered into the parenchyma. Immunostaining for both EBV and CMV was negative. Following drug withdrawal the clinical condition of the patient progressively improved together with the normalisation of liver function tests which occurred after 8 wk of follow-up. Thyroid testing remained in the normal range.

DISCUSSION

We reported two cases of severe PTU-induced hepatitis, one appearing 5 mo after the initiation of therapy and the other one after 6 mo. Liver histology of the first case showed multilobular necrosis, the disease ran a subfulminant course complicated by hepatic encephalopathy and liver failure. In the second case characterised by morphological features of centrilobular necrosis, the clinical and biological evolution was less severe, but ran a prolonged disabling course. The diagnosis of PTU-induced hepatitis was based on the occurrence of liver damage within the first few months of therapy, the absence of previous history and/or other causes of liver disease and the recovery following its discontinuation.

Changes in liver function tests occurring in hyperthyroidism have been known for a long time. Abnormal liver function tests can be observed in 15%-76% of the cases. The pathophysiology of these changes remains unknown, likely involving multifactorial factors including liver congestion, increased liver oxygen consumption, reduced bile acid synthesis, and/or glucuronyl-transferase inhibition^[3,7]. In this setting liver histology only shows non-specific changes^[8].

On the contrary, PTU-induced acute hepatitis occurs in 0.1%-1.2% of treated patients^[4,9,10]. This rate is much higher than that observed in the large context of liver immuno-allergic toxicity (1/10 000 to 1/100 000 or even to 1/1 000 000 for the majority of drugs)^[11], and much lower than that predicted by an asymptomatic increase in ALT which is observed in about 14%-28% of cases during the first week of therapy^[2,10]. In the presence of acute hepatitis the mortality may be as high as 25%^[12] and so far only 28 lethal cases have been reported in the English literature^[6], with two additional cases successfully transplanted^[12,13]. As in our case reports, histological features liver necrosis of variable severity with or without cholestasis^[10], suggesting the role of an immuno-allergic type of toxicity irrespective of the doses of drug used^[5].

In our cases, the delay between the initiation of therapy and the occurrence of symptoms of hepatitis was 5 and 6 mo, respectively. This is in agreement with previous reports^[3,14,15]. The majority of cases are however observed during the first 6 mo of therapy^[11]. The positivity of antineutrophil cytoplasmic antibody (ANCA) testing is not surprising since PTU is a potential causative agent of ANCA positive vasculitis^[16].

In our first observation thiamazol was substituted with PTU without side effect as in another reported case in which carbimazole was used as a substitute to PTU^[17]. Convincing descriptions of thiamazol and PTU hepatic cross-toxicity are indeed lacking, even if cross-sensitivity may occur^[18] and if the rate of untoward cross-reaction between carbimazole and PTU can reach 15%^[18].

In conclusion, this report reinforces the warning against the potential for PTU to induce severe life-threatening hepatitis. The incidence of transient increase in liver function tests at the initiation of treatment reaches about 28%, while the incidence of severe toxicity is about 1%, a risk being much higher than that observed in the majority of compounds considered as potential hepatotoxins. Due to this high incidence of liver toxicity and the rather short delay between the initiation of treatment and the occurrence of hepatitis as well as its relatively slow “subacute” progression, ALT monitoring may be cost-effective in reducing the severity of the liver side effect. The duration of optimal biochemical follow-up remains to be further evaluated in large prospective trials but should be about six months, during which the highest rate of PTU-induced hepatitis is observed.

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Acute coronary syndrome after infliximab therapy in a patient with Crohn's disease

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Abstract

Infliximab is a potent anti-TNF antibody, which is used with great success in Crohn's disease patients. Since its release in clinical practice, several adverse reactions have been observed. The interest in possible consequences of its administration is still high because of the recent introduction of the drug for the long-term maintenance therapy of refractory luminal and fistulizing Crohn's disease. We present a case of acute coronary syndrome (non-STEMI) in a patient with corticoid resistant Crohn's disease after his first dose of infliximab. By reviewing the scant articles that exist in the literature on this topic we made an effort to delineate the possible mechanisms of this phenomenon.

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Key words: Infliximab; Adverse reactions; Crohn's disease; Myocardial infarction; Ischemic heart disease; TNF- α

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INTRODUCTION

Serious adverse events (infections, malignancies, serum sickness, lupus syndrome) that need hospitalization have been reported with a divergent frequency of 6%-18.9% for patients with inflammatory bowel disease (IBD) treated with infliximab^[1,2]. We present a case of acute coronary syndrome (non-ST myocardial infarction) that occurred

after infliximab administration in a patient with corticoid-resistant Crohn's disease.

CASE REPORT

The patient was a 40-year-old male Caucasian who visited the emergency department of our hospital because of chronic diarrhea with 10 watery bowel movements per day. After admission, the patient's history, symptoms, physical signs, laboratory, endoscopic and pathological findings during an extensive diagnostic work up, were compatible with the diagnosis of ileocolonic Crohn's disease (CDAI score: 320). The patient was started on mesalamine orally (1 g t.i.d), metronidazole (500 mg t.i.d) and prednisolone (25 mg b.i.d) intravenously along with parenteral fluids. Five days later 15 mg of prednisolone as well as budesonide enemas (2.3 mg b.i.d) were added to the treatment regimen. The unremitting course of his clinical status on the 12th d of hospitalization was the reason to administer infliximab (5 mg/kg) at a total dose of 375 mg. The infusion of infliximab was well tolerated without any acute adverse reactions. On the first day of treatment the patient's laboratory findings were the following: Ht: 31%, leucocyte count: 9900 (normal), platelets: 314000, CRP: 143 mg/Lt and all biochemicals including basic coagulation tests [prothrombin time (PT), partial thromboplastin time (PTT), international normalized ratio (INR)] were within normal values except serum glucose: 146 mg/dL. Chest X-ray showed only a slightly elevated cardiothoracic ratio. The next day azathioprine 50 mg t.i.d po was added to the therapeutic regimen and prednisolone was reduced to 40 mg iv. On the third day after infusion the patient experienced pain in the chest. The electrocardiogram showed T-wave inversion at the V4, V5, V6 precordial leads and at I, II limb leads with sinus rhythm. Cardiac enzymes [creatinine phosphokinase, creatinine phosphokinase isoenzyme MB, aspartate aminotransferase, lactate dehydrogenase, troponine] related to myocardial infarction were measured and troponine I was found increased: 2.2 ng/mL (normal: < 2 ng/mL). The patient was transferred to the emergency coronary care unit where a diagnosis of acute coronary syndrome implying non-STEMI myocardial infarction was made. During his stay he also suffered an episode of atrial fibrillation, which was reset by amiodarone. He received metoprolol, nitrates, low molecular weight heparin and clopidogrel, which permitted him to recover and return to our Unit after 4 d. Fifteen days after the administration of infliximab the patient's clinical and laboratory status has been improved, with 3 bowel movements per day, absence

of abdominal pain, better general state of health, CRP: 106 mg/Lt and CDAI score: 160.

The patient had no family or personal history of heart disease, hypertension, hyperlipidemia, diabetes mellitus, smoking or obesity and was not taking any drugs before his admission. The physical examination of the heart and ECG at the time of presentation was normal. Fifteen days after the coronary episode the patient was subjected to echocardiography, which showed hypokinesia of the interventricular septum and overall impaired systolic function of the heart with an ejection fraction of 45%. A triplex of the carotids and vertebral arteries did not reveal any atherosclerotic lesions. Our patient also had elevated fibrinogen level (6.55 g/L, normal: 2-4.5 g/L), low protein S activity (41.9%, normal: 75%-130%, Protein S Ac test by Dade Behring), abnormal resistance to activated protein C (APCR) (PCAT: 66.6 s, normal: 85-200 s, Pro C Global protocol assay by Dade Behring) and normal antithrombin III and D-dimers levels under mild systemic inflammation with CRP levels of 32.5 mg/Lt. One month later a cardiac exercise stress test was performed and found normal. Protein S activity and APCR were also normal at that time.

DISCUSSION

It is already known that thromboembolic events occur more frequently in IBD patients (up to 8%) than in normal controls (up to 2%)^[3]. Arterial thrombosis is an uncommon feature of IBD in relation to the far more frequent venous thrombosis. One third of IBD patients develop thrombosis while being in remission, weakening the hypothetical association of active inflammation and abnormal coagulopathy. There are a number of acquired risk factors related with IBD that may predispose to thrombosis. These include immobility, surgery, central venous catheters, parenteral feeding, deficits of vitamins B12 and folic acid as well as hyperhomocysteinemia, increased lipoprotein (a) and anticardiolipin antibodies. Inherited thrombophilic factors like V Leiden and G20210A prothrombin gene mutations have also been implicated in the thrombogenesis of IBD patients. Our patient had none of the above-acquired risk factors as specific investigation of the lipid panel, vitamin B12, folic acid, homocysteine and antiphospholipid antibodies (anticardiolipin, lupus) rendered normal results. The patient was also not subjected to any surgical intervention or parenteral nutrition and because of his young age and self-supporting clinical status was relatively active and not totally immobile. Our patient was also examined for inherited thrombophilic mutations with negative results. It is common for IBD patients to demonstrate abnormal laboratory values of thrombophilic factors in as many as 60% of the cases, a proportion far surpasses the prevalence of thromboembolism in IBD estimated at 8%^[4]. In a study of IBD patients and healthy controls, although 21.4% and 9.5% of patients had reduced free protein S and abnormal APCR respectively, only 4.8% (4 patients out of 84) had a history of thromboembolism and furthermore in two of the patients with thrombosis there were none thrombophilic abnormalities detected^[5]. The abnormal APC resistance in our patient is probably due to the low levels of activated protein S as the patient was not carrying the fac-

tor V Leiden mutation. High plasma fibrinogen level has been associated with increased risk of vascular and non-vascular mortality in a recent meta-analysis study including prospective trials with baseline fibrinogen values and subsequent monitoring of adverse events during at least 1 year of follow up^[6]. The association of increased fibrinogen level with nonvascular events (mainly cancer) weakened the specificity of association with vascular diseases.

Among the other drugs administered to the patient, only steroids have been reported to be associated with coronary heart disease, either myocardial infarction or coronary spasm. These adverse events are related to treatment schedules either with long-term high dose steroid administration (120 mg depomethylprednisolone im for 2 years) or to bolus infusion of high dose steroids (as high as 250 mg methylprednisolone). Our patient was given only 40 mg of prednisolone daily divided into two doses. On the other hand, steroids as well as mesalamine are considered to confer an anti-coagulant effect in patients with Crohn's disease^[7]. This protective role derives from the reduction of proinflammatory cytokines, namely the platelet-activating factor (PAF), TNF- α and IL-1.

Our hypothesis is that infliximab administration might be the cause of the myocardial infarction in this patient. There are three case reports that refer to patients with Crohn's disease (CD) who had venous adverse reactions related to infliximab. The first has reported a patient with retinal vein thrombosis^[8], the second an extensive forearm deep venous thrombosis^[9] and the third comes from a cohort study reporting deep venous thrombosis as well^[2]. It has already been noted that infliximab can also aggravate heart failure if given at a dose of 10 mg/kg to patients with moderate or severe chronic heart failure^[10]. Infliximab has also been found responsible for new onset heart failure in patients with rheumatoid arthritis (RA) or CD, some of them aged below 50 years. An acute coronary syndrome has been described in a patient with RA after infliximab therapy by Abedin *et al*^[11]. However, there are substantial differences in the patient's clinical setting in comparison to our report. The patient in the aforementioned report was female and had a long-standing history of refractory RA starting 20 years ago and a 3-year history of hypertension. She needed multiple therapeutic interventions with corticosteroids, methotrexate, and leflunomide which is known to aggravate hypertension and lastly infliximab. The patient was also obese (95 kg) if we can postulate her weight from the infliximab dose (400 mg total dose, 4.2 mg/kg). Finally, the patient had multiple infliximab infusions in the last 2 years and had already received over 5 g of the drug in escalating dosage. It is obvious that an obese patient with hypertension and an active RA for several years, a disease, which by itself confers vascular compromise, is in danger for cardiovascular events even in the absence of infliximab administration, which already had been given in multiple doses without adverse events in the past. Our patient was a young male with newly presented Crohn's disease who suffered a non-ST myocardial infarction three days after his first dose of infliximab and without having any cardiovascular events in his medical history.

Although TNF has procoagulant properties mainly in high circulating concentrations it seems that during a re-

stricted production it may have antithrombotic activity^[12] by reducing the expression of adhesion molecules like P selectin and the aggregation of platelets as well as their binding to fibrinogen. It has been found that TNF assists in the maintenance of myocardial vascular perfusion by producing vasodilation through the induction of nitric oxide (NO), which is also capable of inhibition of apoptosis of myocardiocytes and attenuation of the heart's stimulation by the sympathetic nervous system through the β -receptors^[10]. TNF is also responsible for the release by myocytes of heat shock proteins (HSP 72, HSP 27)^[13] that are considered to confer resistance against hypoxic stress as well as for the production of manganese superoxide dismutase, a scavenger of free radicals^[14]. The administration of a potent anti-TNF antibody like infliximab, which neutralizes both soluble and membrane TNF can abrogate these homeostatic interventions causing deprivation of first line defensive mechanisms. Infliximab has been shown to cause vasoconstriction and increased wall shear stress in arteries of patients with RA after a dose of 3 mg/kg^[15].

On the other hand, there are several reports of unaffected serum TNF- α concentrations despite administration of anti-TNF antibodies in patients with either RA, leucoclastic vasculitis or after myocardial ischemia. High levels of TNF- α in the serum have been associated with higher risk for myocardial infarction (OR: 1.7). It has been found in experimental models that endogenous production of TNF can downregulate its relevant receptors and therefore confer resistance to its continuous action. A counter-regulatory mechanism might upregulate the expression of mTNF and TNF-receptors on the cell membrane of endothelium, monocytes, vascular smooth muscle cells (VSMCs) and myocytes, at the time when anti TNF administration causes a reduction of the serum or tissue TNF levels, with the result of a rupture plaque or myocardial apoptosis. An increase in serum TNF- α levels has been noted in two studies of patients with RA and chronic heart failure respectively after the initial drop in TNF concentration following administration of infliximab^[10,16]. In the former study the increase was noted on the 3rd till the 7th d after infusion. The authors inferred that TNF- α was not bioactive but rather was bound to anti-TNF antibodies. Because of the inability of TNF- α assays to measure free TNF, one can surmise that this paradoxical increase may not be so innocent and could activate monocytes to produce metalloproteinases or stimulate endothelial cells to become thrombogenic by releasing NO, thromboxane and platelet adhesion molecules.

There is therefore a need to study the TNF-receptor expression in Crohn's disease in the myocardium. Furthermore the study of TNF polymorphisms could offer an explanation for the susceptibility of some individuals to the cardiac effects of anti-TNF- α .

In conclusion, we support the notion that anti-TNF therapy is the "probable" cause of acute coronary syndrome in this patient because of the chronological sequence between its administration and the presentation of this adverse reaction (3 d). We also demonstrated that the drug was active for the patient's clinical condition and that the individual completely recovered one month later

when the apparent effect of the drug was completely abolished. We could not find in the English literature an absolutely proven causal relationship between the pathogenesis of Crohn's disease and thromboembolic events. According to published criteria for the estimation of the probability of adverse drug reactions, this reaction could be rated as "probable"^[17].

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A case of solid pseudopapillary tumor of the pancreas

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Abstract

We present ultrasound, computed tomography and magnetic resonance imaging findings in a case with pancreatic solid pseudopapillary tumor and their correlations with histopathology. Ultrasound revealed a hypoechogenic mass, and computed tomography revealed a hypodense mass at the pancreatic head minimally enhanced after intravenous contrast agent administration. Magnetic resonance imaging showed a hypointense mass on unenhanced T1-weighted images including a hyperintense focus representing the hemorrhage. The lesion was hyperintense on T2-weighted images. On the postcontrast images the lesion showed peripheral thin contrast enhancement in arterial phase and enhanced slightly diffusely in venous and equilibrium phases. The patient underwent elective resection of the mass and pancreatoduodenectomy with jejunostomy tube placement. A final diagnosis of solid pseudopapillary tumor was made histopathologically. Solid pseudopapillary tumor is a rare pancreatic tumor. It is important to make the diagnosis preoperatively because with an adequate surgical resection the prognosis is good. A multimodal approach, especially magnetic resonance imaging can suggest the diagnosis without the need for biopsy.

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Key words: Pancreas; Solid pseudopapillary tumor; Magnetic resonance imaging; Computed tomography; Ultrasound

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INTRODUCTION

Most pancreatic tumors are malignant and have a bad prognosis. However, solid-pseudopapillary tumor of the pancreas (SPT) is a rare benign or low-grade malignancy. Because of this, it is important to make the diagnosis of this tumor preoperatively so that adequate resection will be undertaken. SPT accounts for less than 1%-2% of the exocrine pancreatic tumors^[1-5]. It affects mostly young females between the 2nd and 3rd decades of life with a male to female ratio of 1:9. The patients with SPT are often clinically asymptomatic or minimally symptomatic^[3,6,7]. Therefore imaging studies should be carefully assessed^[1,2]. Ultrasonography (US), computed tomography (CT) and magnetic resonance (MR) imaging are the useful modalities in the diagnosis of SPT which is not uncommonly diagnosed incidentally at abdominal imaging^[7]. Here we report a case of a 17-year old girl presenting with this rare pancreatic tumor and discuss the differential diagnosis from other pancreatic masses.

CASE REPORT

A 17-year old woman complaining of dyspeptic symptoms and right upper quadrant pain for several months was admitted to our hospital for further evaluation. She had no history of abdominal trauma or surgery, drug usage or smoking. On physical examination tenderness in the right upper quadrant and epigastrium was noted. Laboratory findings showed no abnormalities.

An abdominopelvic US revealed a heterogenous hypoechogenic solid mass lesion at the pancreatic head region (Figure 1). CT scan showed a round shaped, well-marginated, hypodense mass at the pancreatic head with a density of 38.19 HU and no evidence of internal hemorrhage (Figure 2). After intravenous iodine contrast agent administration, the lesion showed minimally heterogenous contrast enhancement markedly less than the normal pancreatic tissue. MR examination included axial fast spoiled gradient-echo (FSPGR) fat saturated T1-weighted images and axial and coronal single-shot fast spin-echo T2-weighted images. Unenhanced and contrast-enhanced arterial, portal venous and equilibrium phase images were obtained. MR showed that the mass lesion was predominantly hypointense on unenhanced T1-weighted images including a hyperintense focus representing the hemorrhagic degeneration (Figure 3). The lesion was predominantly hyperintense (but less



Figure 1 A hypoechoic well-margined solid mass is seen at the pancreatic head region (arrows). Vena cava inferior is slightly compressed by the mass. SV: Splenic vein; P: Pancreatic tail.

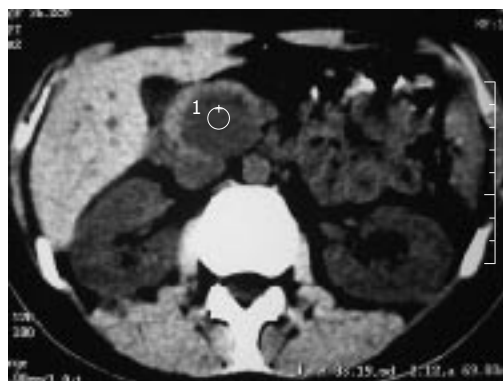


Figure 2 Axial precontrast CT image shows that the mass is hypodense with no evidence of calcification. The density of the mass is measured 38.19 HU.

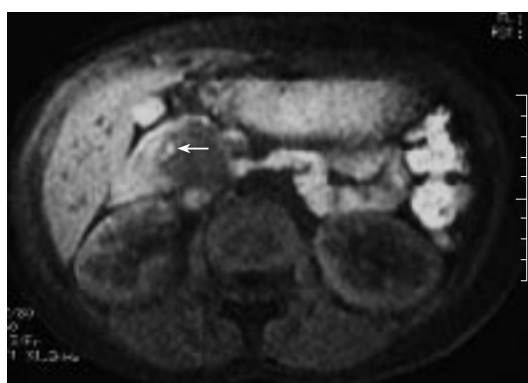


Figure 3 Precontrast T1-weighted axial MR image shows that the mass is predominantly hypointense and the hyperintense focus represents hemorrhage (arrow).

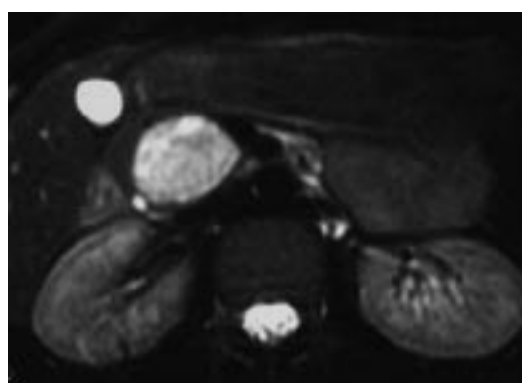


Figure 4 T2-weighted axial MR image shows that the mass is predominantly hyperintense mostly composing of solid compounds and minimal cystic areas.

than cerebrospinal fluid) on T2-weighted images mostly composed of solid and minimally cystic areas (Figure 4). On the initial arterial-phase contrast-enhanced images the lesion was predominantly hypointense with some peripheral thin contrast enhancement (Figure 5A). On the portal venous and equilibrium phase images the lesion seemed to enhance diffusely slightly (Figure 5B and 5C).

The patient underwent elective resection of the mass in the pancreatic head and pancreaticoduodenectomy (Whipple procedure) with jejunostomy tube placement. The resection material was sent to the pathology department. At gross examination, a well-circumscribed, capsulated, round, gray-purple colored tumor was identified in the pancreatic head. By histologic analysis, pseudopapillary aggregates including stromal central vessels were detected. They were separated with partially defective thin fibrous capsule from the pancreatic tissue leading to some pancreatic invasion locally. The tumor was composed of uniform polygonal cells with prominent nucleolus, uniform nucleus and narrow eosinophilic cytoplasm (Figure 6A and 6B). No vascular space or perineural invasion was identified. By immunohistochemical analysis, the tumor cells were positive for vimentin and synaptophysin, and for progesterone receptors (Figure 6C); and negative for cytokeratin, chromogranin and estrogen

receptors. In the evaluation of Ki-67, less than 5% of the tumor cells showed nuclear proliferative activity. These are compatible histopathologic findings for SPTs except for synaptophysin positivity which is unusual.

After the surgery the patient underwent MR imaging examination per 6 mo for the first year and then once a year for the following 2 years until the present and no recurrence, metastases or symptoms occurred.

DISCUSSION

SPT is a rare pancreatic tumor consisting of 1%-2% of exocrine pancreatic tumors^[1,7]. The precise incidence of SPT is not known because it is rare and is frequently misdiagnosed^[1]. This tumor was first described by Frantz in 1959 as a “papillary tumor of the pancreas, benign or malignant”^[8]. Since then, various names have been used to describe this rare lesion, such as solid and cystic tumor of the pancreas, papillary-cystic tumor, solid and papillary epithelial neoplasm and Frantz tumor^[1,2,7]. In 1996, the World Health Organization (WHO) renamed this tumor as SPT for the international histologic classification of tumor of the exocrine pancreas^[7,9].

SPTs are benign lesions but sometimes malignant degeneration may occur especially when the lesion occurs

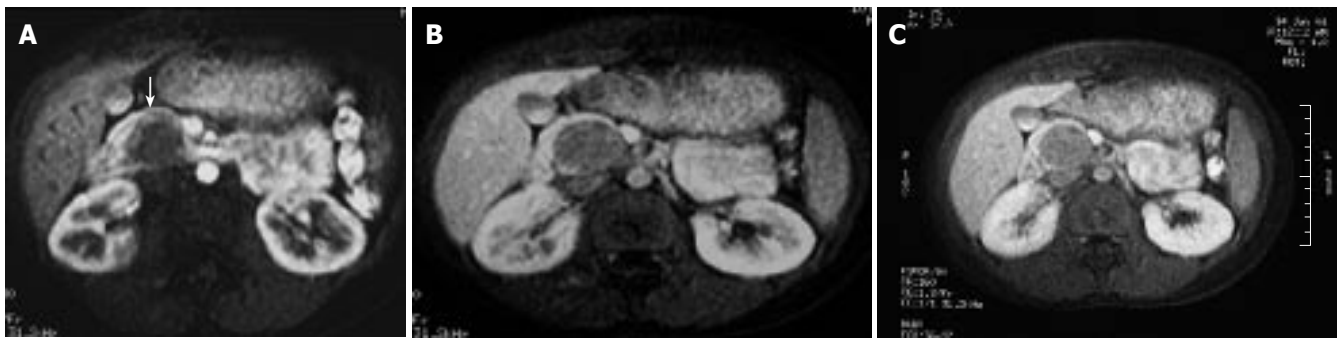


Figure 5 A: Arterial-phase contrast-enhanced T1-weighted image shows minimal peripheral thin contrast enhancement (arrow); B, C: Portal venous and equilibrium-phase contrast-enhanced T1-weighted images show that the mass enhances diffusely slightly.

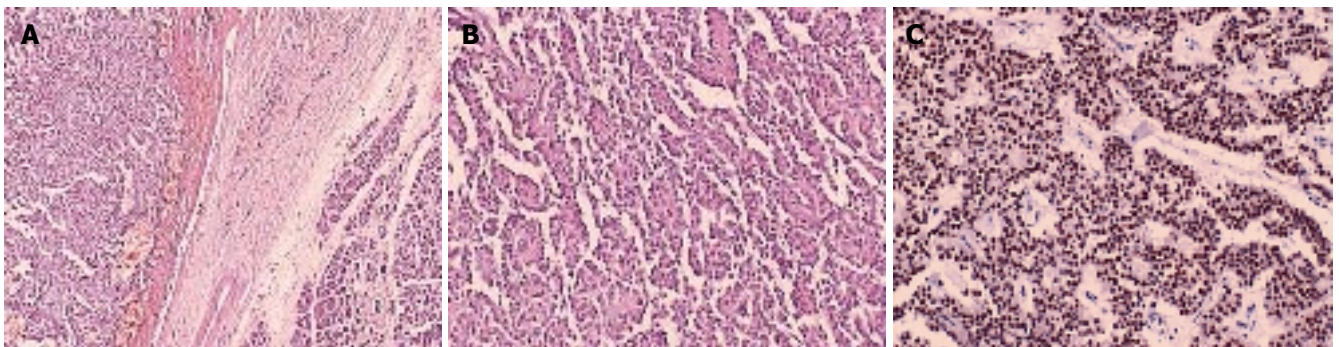


Figure 6 A: Pancreatic tissue is seen around the tumor including papillary structures and hemorrhagic areas (HE \times 40); B: Solid pseudopapillary tumor. Pseudopapillary aggregates lined by tumor cells with uniform nucleus and narrow eosinophilic cytoplasm are visible (HE \times 40); C: Micrograph shows progesterone receptor positivity in the nucleus of tumor cells (\times 100).

in elderly male patients^[2,7].

SPT of the pancreas has distinctive pathologic features. The mass is most frequently found in the head or tail region. On gross examination, the mass is usually large and well encapsulated and contains varying amounts of necrosis, hemorrhage, and cystic change. By microscopic analysis, there are two distinct types of cellular arrangements: solid and papillary^[7]. The pathogenesis of these tumors is still controversial. Whereas some authors postulate an endocrine origin, others claim these tumors arise from ductal cells, acinar cells, or pluripotent stem cells^[1]. Vito *et al*^[1] claimed that, SPT was associated with pregnancy in 3, with polycystic ovary disease in 2, with Sertoli-Leydig cell tumor in 1 of their 19 patients, which supports an endocrine origin for SPT of pancreas^[1]. Also a hormonal influence is also suggested in view of the tumor's high prevalence in women.

SPTs are typically positive for vimentin, neuron-specific enolase (NSE), α -1-antitrypsin, and α -1-antichymotrypsin and negative for chromogranin A, epithelial membrane antigen, and cytokeratin. Differentiation along endocrine cell lines has been postulated for this tumor on the basis of NSE positivity, but the expression of vimentin and α -1-antitrypsin does not support this interpretation^[7]. A study by Kosmahl *et al*^[10] demonstrated that SPTs have a complex immunoprofile that is inconsistent with that of any of the pancreatic cell types and that a pancreatic origin is unlikely. The authors speculated that on the basis of some similarities between SPT and ovarian surface cells

and the proximity between genital ridges and the pancreas anlage during early embryogenesis, SPTs might originate from the genital ridge-related cells that were incorporated into the pancreas during organogenesis. Furthermore, sex hormones may play a role in the pathogenesis or growth of SPTs: Nearly all studies demonstrate no evidence of estrogen receptors; however, progesterone receptors are present in many cases^[7,11] as presented in our case.

The origin of SPTs has not yet been clarified. SPTs cannot be regarded as a purely endocrine neoplasm because the presence of neuroendocrine markers is not significant (chromogranin A is not detected, synaptophysin has a patchy immunoreactivity in 22% and NSE is strongly positive in more than 90% but without a certain significance)^[12-14].

However, SPTs are classified and are generally held to be epithelial neoplasms but immunohistochemical patterns suggest that SPTs cannot be regarded as purely epithelial neoplasms^[12,15]. In fact, cytokeratin expression, usually associated with epithelial differentiation, is rare (less than 30%) or absent in most cases, and markers of acinar differentiation (trypsin/chymotrypsin) and glycoprotein markers of ductal differentiation are often negative^[12,16,17].

Although all these hypotheses of origin are intriguing, they are yet unproved, and further research is necessary.

Another interesting point is the relationship between the synaptophysin positivity and recurrence rates of SPTs. Lai *et al*^[18] analysed 7 patients histopathologically and found that 6 of the patients with no recurrence were negative

for synaptophysin and 1 of the patients who suffered from recurrence was positive. In our case synaptophysin was unusually positive with no recurrence after surgical management in the period of 3 years until now.

Patients with SPT are often clinically asymptomatic^[2,3,6,7]. They may present with palpable abdominal mass, abdominal pain or discomfort^[1-3,6,7] and also acute abdomen can develop due to the rupture of the lesion^[2,3,19]. The abdomen is usually nontender on palpation but obstructive symptoms may occur if the tumor grows large enough to compress adjacent anatomic structures^[7]. However, bile duct or pancreatic duct obstruction is rare, even when it is located in the head of the pancreas because of its softness^[1]. Physical examination is often normal apart from the presence of an upper abdominal mass^[2], and usually no laboratory abnormalities appear associated with pancreatic insufficiency, abnormal liver function, cholestasis, elevated pancreatic enzymes, or an endocrine syndrome. Tumor markers are also expected to be normal^[2,7]. Also an unusual presentation of SPT with acute pancreatitis was reported in the literature^[6].

Given the good prognosis of the disease, it is important to make the diagnosis preoperatively so that adequate resection will be undertaken. Therefore imaging studies should be carefully assessed^[1,2]. US, CT and MR are the useful diagnostic modalities in the diagnosis of SPT which is not uncommonly diagnosed incidentally at abdominal imaging^[7].

On US examination the lesion is well-encapsulated and includes cystic and solid components, but sometimes the mass is pure and solid-looking or has internal septa or calcifications^[7]. Usually the appearance of SPT is variable and lacks correlation with gross pathology^[1].

Contrast-enhanced CT plays a major role in the diagnostic evaluation of cystic neoplasms of the pancreas. However, when compared with MR imaging, CT has inherent limitations in showing certain tissue characteristics, such as hemorrhage, cystic degeneration, or the presence of a capsule. These features may, as shown at pathology, be suggestive of specific lesions such as SPT of the pancreas. Therefore, MR imaging may further aid in showing these characteristics and in the differential diagnosis of complex cystic masses within the pancreas^[1].

CT usually demonstrates a well-encapsulated, heterogeneous lesion with varying solid and cystic components^[7]. After intravenous contrast agent administration, enhanced solid areas are typically noted peripherally, whereas cystic spaces are usually more centrally located^[7].

MR imaging, because of its superior contrast resolution, displays a capsule and intratumoral hemorrhage better than CT. Therefore, MR imaging has the potential to improve our ability to diagnose SPT^[1]. MR images demonstrate a well-defined lesion with heterogeneous signal intensity on T1 and T2-weighted images, which reflects the complex nature of the mass^[7]. In previous reports all SPTs contain some high signal intensity on T1-weighted images representing the blood products^[1,7]. A hyperintense focus was also noted on T1-weighted axial images of our case. Vito *et al*^[1] also defined that 5 of 19 patients did not show high signal on T1-weighted images in their study. Therefore, the absence of high T1 signal should not

exclude the diagnosis.

The T2-weighted appearance was described differently in the literature. Ohtomo *et al*^[20] reported findings for six patients: the masses appeared hyperintense in four patients and mixed—that is, hyper- and hypointense—in two patients. Buetow *et al*^[21] reported areas of high signal intensity on T2-weighted images in nine patients and areas of low signal intensity in three patients. However, they did not report the predominant signal intensity of the masses or whether the lesions appeared homogeneous. Vito *et al*^[1] reported findings for 19 patients: 14 lesions (73.5%) appeared heterogeneous, and all lesions but two (89%) appeared predominantly hyperintense on T2-weighted images. In our case the lesion was also predominantly hyperintense on T2-weighted images.

Angiography usually demonstrates an avascular or hypovascular pancreatic tumor and may help delineate the mass from other involved and adjacent structures^[2]. Vito *et al*^[1] reported that most common enhancement pattern of SPT consists of early, peripheral, and heterogeneous enhancement during the arterial phase with progressive but heterogeneous fill-in of the lesion during the portal venous and equilibrium phases. They also reported that all but one lesion in their series enhanced less than the adjacent normal pancreas during all phases. This feature helps distinguishing SPT from other pancreatic neoplasms, such as islet cell tumors, which typically enhance more than the pancreas. Vito *et al*^[1] reported that all but one of their patients had a peripheral hypointense tumor capsule on both T1- and T2-weighted images. Compared with the lesion, the tumor capsule showed early and more intense enhancement (70%), identical enhancement (20%), or less enhancement (10%). Ohtomo *et al*^[20] reported that four patients (67%) presented with a fibrous capsule detected as a band of low signal intensity on T1- and T2-weighted imaging. Conversely, Buetow *et al*^[21] observed in all their patients a discontinuous low-signal-intensity rim on T2-weighted images. In our case a capsule formation was not detected.

Serous microcystic adenoma, mucinous cystic neoplasm, cystic islet cell tumor, pancreaticoblastoma, and calcified hemorrhagic pseudocyst are differential diagnostic considerations when a pancreatic mass consists of cystic and solid components. The former three tumors occur rarely in patients younger than 30 years^[1,22]. On T1-weighted MR images, serous microcystic adenoma may appear as a heterogeneous hypointense mass with hyperintense foci related to prior hemorrhage and could be confused with SPT. However, on T2-weighted images, serous microcystic adenoma shows hyperintense signal intensity with low-signal-intensity central areas due to scar formation and hypointense septa that may radiate toward an enhanced central scar^[1,22,23]. This radial central scar is not a feature of SPT^[1].

Mucinous cystic neoplasms may be confused with SPT, particularly when the SPT is predominantly cystic^[1,22,23]. Although the mucin-filled cystic spaces are typically hyperintense on T2-weighted images and hypointense on T1-weighted images, mucin occasionally results in high signal intensity on both T1- and T2-weighted images^[1,23]. The signal intensity may vary depending on the

proteinaceous content of the mucin. Also, mucinous cystic neoplasms do not exhibit early peripheral and capsular enhancement^[1].

Islet cell tumors occur in patients who are older and do not have the female predominance. They may appear cystic, contain calcifications, and show areas of internal hemorrhage^[1,21,24]. Islet cell tumors are low in signal intensity on fat-suppressed T1-weighted images, exhibit high signal intensity on T2-weighted images, and show marked ring or diffuse heterogeneous enhancement on immediate gadolinium-enhanced gradient-echo images^[1,21]. The differential diagnosis of SPT from islet cell tumor of the pancreas is the different signal intensity on T1-weighted images and excess contrast enhancement of islet cell tumors after gadolinium injection due to their more vascularity compared with SPTs^[1].

Pancreatoblastoma is more aggressive than SPT and often presents with liver metastases at the time of diagnosis. Intratumoral hemorrhage has not been reported in these tumors using either CT or MR imaging. Although pancreatic adenocarcinoma is the most common primary pancreatic malignancy, it is seen in older patients. Pancreatic pseudocysts may be calcified peripherally as a result of internal hemorrhage and may mimic SPT. However, a history of pancreatitis is almost always present, and pancreatic pseudocysts can be distinguished from SPT by the absence of solid components^[1].

In conclusion, when a young woman presents with a pancreatic mass lesion, SPT must be considered in the differential diagnosis. Multimodal approach, especially MR could suggest the diagnosis of SPT with some imaging features including well-marginated, encapsulated, solid-cystic mass with areas of hemorrhagic degeneration and progressive peripheral or heterogeneous contrast enhancement.

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Ian David Wallace, MD

Shakespeare Specialist Group, 181 Shakespeare Rd, Milford, Auckland 1309, New Zealand



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
isvhl2006@mci-group.com
www.isvhl2006.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
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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
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c.chase@imedex.com

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Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology
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Society of American Gastrointestinal
Endoscopic Surgeons
26-29 April 2006
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Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
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American Society of Gastrointestinal
Endoscopy
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American Society of Colon and Rectal
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3-7 June 2006
Seattle - Washington
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EVENTS AND MEETINGS IN 2006

10th World Congress of the International
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Week
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European Multidisciplinary Colorectal
Cancer Congress 2006
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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 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]

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- 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]

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- 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>

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- 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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Quality of life in chronic pancreatitis

Raffaele Pezzilli, Laura Bini, Lorenzo Fantini, Elena Baroni, Davide Campana, Paola Tomassetti, Roberto Corinaldesi

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Abstract

In an era such as the present one in which there is a high demand for health services with the associated pressure of controlling spending, health care organizations are concerned about the cost-effectiveness of quality improvement interventions. On the other hand, the impact of the disease and the treatment on the patient's overall well-being and functioning has become a topic of growing interest not only in clinical research but also in practice. The clinical evaluation of the benefits of specific treatments for chronic, debilitating and incurable diseases should increasingly include formal assessment of patient activity and well-being. Thus, health-related quality of life as subjectively perceived by the patient, is becoming a major issue in the evaluation of any therapeutic intervention, mainly in patients with chronic or difficult diseases where the aim of the intervention is to keep patients either symptom-free and capable of living in the community for a long time or to reduce the discomfort caused by the disease. In this paper, we review the current knowledge on the quality of life assessment in chronic pancreatitis patients.

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Key words: Pancreatitis; Pancreatitis; Alcoholic; Quality of Life; Questionnaires

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INTRODUCTION

In the present era, there is a high demand for health services with the associated pressure of controlling

spending, health care organizations are concerned with the cost-effectiveness of quality improvement interventions. On the other hand, the impact of the disease and the treatment on the patient's overall well-being and functioning has become a topic of growing interest not only in clinical research but also in practice. The benefits of the specific treatments as well as the health-care system will be judged more and more on the basis of how the changes in the patient's activity or well-being correspond to his or her expectations. Thus, health-related quality of life as subjectively perceived by the patient, is becoming a major issue in the evaluation of any therapeutic intervention, mainly in patients with chronic or difficult diseases where the aim of the intervention is to keep patients either symptom-free and capable of living in the community for a long time or to reduce the discomfort caused by the disease. Several questionnaires have been developed to measure the health-related quality of life with the number of items ranging from 10 to 100; some of the questionnaires are designed for the assessment of a non-disease group while others were developed for specific disease groups.

CHRONIC PANCREATITIS

Chronic pancreatitis is a disease which is often characterized by recurrent episodes of abdominal pain accompanied by progressive pancreatic exocrine and endocrine insufficiency, and it sometimes requires multiple hospitalizations. The disease is frequently the result of chronic alcohol abuse even if other etiological factors such as genetic alterations, autoimmune disorders and obstructive disease of the biliary tract and the pancreas have recently been postulated. The management of chronic pancreatitis remains a challenging puzzle; for most patients, medical treatment is a good option, especially in those requiring substitutive therapy for either exocrine or endocrine insufficiency; however, controlling the pain remains the main therapeutic challenge. Although the medical management of pain may be one of the therapeutic modalities^[1], in the past as well as in the present, surgical management has been the main option in the case of intractable pain^[2]. In recent years, other therapeutic options, more medical than surgical, have been applied in clinical practice: endoscopic therapy^[3], thoracoscopic splanchicectomy^[4], and extracorporeal shockwave lithotripsy^[5]. The mechanism of pain in chronic pancreatitis is certainly multifactorial and seems to be mainly based on two mechanisms: ductal hypertension, and

neural and perineural inflammation. The medical option is generally the first which is attempted based on NSAIDs and often on opioids but it is not always satisfactory and, before narcotic dependence develops, endoscopy or surgery should be considered. Endoscopic and surgical treatment is generally the first option when there is any cause of ductal hypertension; however, a surgical approach remains the main option for intractable pain. Other complications in patients with chronic pancreatitis are the formation of pseudocysts, fistulae, bleeding due to rupture of esophageal and/or gastric varices secondary to splenic or portal thrombosis, biliary and duodenal strictures, ascites, and superimposed carcinoma. All these complications require a multi-specialist approach, even if, in most cases, surgery remains the only option.

QUALITY OF LIFE EVALUATION

In the past few years, several self-administered questionnaires for the assessment of the quality of life have been developed. All the questionnaires have been constructed to evaluate two main domains: physical and mental well-being. The items contained in the various questionnaires vary greatly (from 6 to more than 130 questions) and the association of the different items permits the calculation of the various domains. Generally, a high score of the various domains corresponds to a good quality of life whereas a low score indicates a poor quality of life.

In recent years, many studies evaluating the quality of life in chronic pancreatitis patients have been published; these studies involved mixed medical-surgical patients^[6-9]. Three of these studies utilized a questionnaire called Medical Outcome Study 36-Item Short-Form Health Survey (SF-36)^[6-8] made up of 36 items and the most recent study, a questionnaire constituted by two different modules, the European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire-C30 (EORTC QLQ-C30), made up of 30 items, and the Quality of Life Questionnaire pancreatic cancer module (QLQ-PAN26), made up of 26 items, which had previously been tested in pancreatic cancer patients^[9].

All four studies demonstrated that patients with chronic pancreatitis have a substantially impaired quality of life and, most importantly, the impairment of the quality of life in younger patients is higher than in older ones with obvious economic consequences for society.

Regarding gender, in the Italian study^[8], the impairment of various domains was more pronounced in females and this finding differs from that of the German studies^[6,7]. This may be explained, at least in part, by the fact that Italian females affected by chronic pancreatitis have a poor acceptance of the disease.

Among the various clinical variables examined as possible factors related to chronic pancreatitis (pancreatic calcifications, pseudocysts, Wirsung, duct dilatation, pancreatic insufficiency, diabetes), only pain was able to significantly impair all eight domains of the SF-36, thus confirming that pain control is the main therapeutic option to be taken into account in order to improve the quality of life in patients with chronic pancreatitis, thereby suggesting that much effort should be made in order to identify more

efficacious therapies capable of controlling this symptom.

Surprisingly, in the Italian study^[8], neither the type of pancreatic surgery nor endoscopic therapy were able to substantially modify the various physical and mental domains investigated by the SF-36; this is in contrast to previous studies regarding the various surgical and endoscopic options^[4,10-18], and such a difference may be due to the fact that these studies enrolled a highly selected group of patients with a short time interval between intervention and the assessment of the health-related quality of life (3-74 mo); another possible bias present in these surgical/endoscopic studies is that their data were not adjusted for sex and age.

It is worth noting that diabetes and major alterations of the Wirsung duct (which are expressions of long-standing chronic pancreatitis), as well as a decreased BMI (which is an expression of maldigestion) are able to impair some of the physical and mental domains^[6-8].

Co-morbidities were not significantly related to the quality of life of these patients^[6,8]; a possible explanation of this phenomenon is the fact that chronic pancreatitis *per se* determines a high impairment of the quality of life and co-morbidities add little, since these patients already had low values for most of the SF-36 domains.

An important point is that a percentage of patients varying from 4% to 10%^[8,9] missed responses or refused to complete the questionnaires. In the Italian study^[8], this group was better characterized; the patients who refused to complete the questionnaire were male patients, current smokers with a long duration of alcohol consumption, with a long duration of the disease, and free from pain at the time of the study. Patients with the above-mentioned characteristics are probably candidates for an intensive psychological approach in order to counter-balance their unwillingness to improve their relationship with the disease.

The main differences in the four studies exploring the quality of life in chronic pancreatitis patients^[6-9] are that the studies utilizing the SF-36 questionnaire had a control group taken from the general population^[6-8] whereas the study utilizing the EORTC QLQ-C30 and the QLQ-PAN26 did not^[9]; the studies utilizing the SF-36 had a wide number of chronic pancreatitis patients coming from the country where the studies were carried out whereas the study utilizing the EORTC QLQ-C30 and the QLQ-PAN26 enrolled 66 patients coming from four different countries (Germany, Italy, South Africa, and United Kingdom); finally, all the patients utilizing the SF-36 questionnaire were fluent in the native language^[6-8] whereas Afrikaans-speaking patients in South Africa completed the English version of the EORTC QLQ-C30 and the QLQ-PAN26. Since in clinical practice, there is the need to utilize a time-saving questionnaire to assess the quality of life, we have recently carried-out a study utilizing a short version of the SF-36 questionnaire named SF-12 (Medical Outcome Study 12-Item Short-Form Health Survey)^[19]. The aim of this study was to establish the validity of the SF-12 questionnaire in patients with chronic pancreatitis and to identify the predictors capable of modifying the physical (PCS) and mental (MCS) summaries in these patients. The SF-12 and the SF-36 questionnaires were used. One hundred and forty-one patients with proven

chronic pancreatitis were studied. The chronic pancreatitis patients had SF-12 physical (PSC-12) and mental component (MCS-12) summaries significantly related to the PCS-36 and MCS-36 ($P < 0.001$). The presence of pancreatic pain and non-pancreatic surgery accounted for 41.3% in the formation of the PCS-36 score and 37.2% in that of the PCS-12 score, respectively. Gender, BMI, and pancreatic pain accounted for 15.3% of the information in the formation of the MCS-36 and for 14.7% in that of the MCS-12; using these clinical variables, the loss of information in applying the SF-12 instead of the SF-36 was very low (4.6% and 0.6% for the PCS and the MCS, respectively). Thus, the SF-12 seems to be a good alternative to the SF-36 in assessing the quality of life in chronic pancreatitis.

CONCLUSION

The conclusions that can be drawn from studies which have assessed the quality of life in chronic pancreatitis patients are the following: it is necessary to choose a widely accepted questionnaire concerning the quality of life in order to render the various studies in different populations of chronic pancreatitis patients comparable and we need further studies comparing the various questionnaires in order to identify the questionnaire which could be the most useful in routinely evaluating our patients in the office. At present, the SF-12 questionnaire is the instrument of choice to assess the quality of life in the doctor's office. The presence of papers assessing the quality of life in chronic pancreatitis patients leads us to believe that all future studies on the management of chronic pancreatitis should include a validated questionnaire in order to evaluate the point of view of the patient on the various treatments employed^[20].

We recommend the routine use of QoL questionnaires to assess the well-being of patients with chronic pancreatitis in order to select those who need a more intensive medical and psychological approach. However, we could couple the QoL assessment with traditional clinical methods to evaluate the therapeutic effects of the various medical approaches.

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EDITORIAL

Techniques for restoring bowel continuity and function after rectal cancer surgery

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methods to improve bowel function very acceptably; the future advances are likely in laparoscopy.

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Abstract

A very low local recurrence rate of 3%-6% (associated with improved 5 year survival) is possible when proper oncological surgery is performed of mid and distal rectal adenocarcinoma. Restoration of bowel continuity is possible in most cases, without compromise of cancer clearance. Re-anastomosis can be performed with stapled, transabdominal hand-sewn or coloanal pull-through techniques. However after a direct (straight) anastomosis of the colon to the distal rectum/anus, up to 33% of patients have 3 or more bowel movements/d; some can be troubled with up to 14 stools a day. Construction of a 6-cm colonic J-pouch is likely to cause some reversed peristalsis which improves postoperative bowel frequency without causing neo-rectum evacuation problems. Colonic J-pouch-anal anastomosis patients have a median of 3 bowel movements a day compared with a median of 6 a day for straight anastomoses, at 1 year after surgery. In the longer term, bowel adaptation may enable the function after a straight anastomosis to approximate that of a colonic J-pouch-anal anastomosis. This probably depends in the former, upon whether the more rigid sigmoid colon or more distensible descending colon is used. An additional advantage of the colonic J-pouch-anal anastomosis is the lower risk of anastomotic complications. A more vascularized side-to-end (colonic J-pouch-anal) anastomosis is likely to heal better than an end-to-end (straight) anastomosis. Where the pelvis is too narrow for a bulky colonic J-pouch anal anastomosis, a coloanal-anal-anastomosis is an option. The latter results in postoperative bowel function comparable with the colonic J-pouch. However, the risk of anastomotic complications is higher possibly related to its end-to-end anastomotic configuration. Laparoscopic techniques for accomplishing all the above are being proven to be effective. Restorative surgery for rectal cancer can be safely and effectively performed with

INTRODUCTION

The most important priority in the surgical management of mid and distal rectal cancers is adequate oncologic clearance. It is generally accepted that this is achievable by total mesorectal excision, although in Japan extended pelvic lymphadenectomy is also used in selected cases. Total mesorectal excision involves precise excision of the entire rectum and para-rectal lymph nodes en-block, within an oncologic package termed the 'mesorectal envelope'. A very low local recurrence rate of 3%-6% (associated with improved 5 years survival) has proven to be repeatable, where the surgeon is adequately trained and the technique is correctly practised^[1-3]. Restoration of bowel continuity after total mesorectal excision is possible most of the time, without compromise of oncologic clearance. The reported mortality of 7%-8% is comparable to the 2.2%-8% following the alternative abdominoperineal resection^[4-6]. Furthermore, local recurrence rates following low anterior resection (7%-14.7%) are similar to that of abdominoperineal resection (12%-18.8%)^[4-6].

Confusion in the literature can be minimized by defining clearly the terminology relating to the restoration of bowel continuity after rectal cancer surgery. The type of procedure is defined by the anatomical site of anastomosis rather than the position of the cancer, as commonly described at colonoscopy. The term "high" anterior resection refers to a colorectal anastomosis performed at the level of between the sacral promontory and the anterior peritoneal reflection. The level of the anastomosis is normally measured to be about 8 to 16 cm from the anal verge, depending upon the patient's body build. The term "low" anterior resection refers to a colorectal anastomosis performed at between distal to the anterior peritoneal reflection and proximal to the anorectal junction. This is

normally measured to be about 5 to 8 cm above the anal verge, again depending upon the patient's body build. The term "ultra-low" or "extended" anterior resection refers to a colorectal or more usually, a coloanal anastomosis at the level of the anorectal junction. This is the type of anastomosis that is performed after proper total mesorectal excision and incision of the Waldeyer's fascia posterior to the rectum. The latter technical step allows the rectum to be mobilized/"freed" both anteriorly and proximally from the pelvis, allowing for transection of the rectum safely at the anorectal junction. The level of this anastomosis is normally measured to be about 3-5 cm from the anal verge. Such distal anastomoses have much higher risk of anastomotic dehiscence and consideration should be given to temporary defunctioning by either a colostomy or ileostomy. The term "intersphincteric dissection" refers to the special situation where a very distal rectal cancer is excised with clear oncological resection margins by including an en-block excision of the internal anal sphincter and anal mucosa, to the level of the dentate (pectinate) line. The last part of the procedure is usually performed transanally. Reconstruction is done by a pull-through procedure with hand-sewn anastomosis of the colon to the distal anus, again by the transanal route.

Subsequent discussion will be along (1) methods of anastomosis after resection of the rectal cancer, (2) types of operations i.e. straight colorectal or coloanal anastomosis, colonic J-pouch anal anastomosis, coloplasty anastomosis & colonic side-to-end anastomosis, and (3) anterior resection syndrome & management.

ANASTOMOTIC METHODS

Following successful resection of the rectal cancer with total mesorectal excision, bowel continuity can be restored by coloanal/distal rectal anastomosis performed using various techniques.

Stapled anastomosis

The most common method of performing the coloanal/distal rectal anastomosis is with the use of a circular intraluminal stapling instrument introduced transanally. It is technically challenging to insert a hand-sewn *purse-string suture* to the transected rectal stump and to secure the shoulder of the transanally inserted stapling device, as the transected anorectal junction tends to retract into the pelvic floor. The exposure is often further obscured by a hypertrophied bladder which is commonly found in the older male patients with bladder outlet obstruction problems such as benign prostatic hypertrophy. Hence, the double cross stapled technique is the most commonly employed method particularly in the West where patients are usually of bigger size^[7]. The double cross stapled technique consists of stapling and transecting the anorectal junction distal to the cancer. Prior to this, it is important to irrigate the rectum with tumoricidal agents to reduce the risks of cancer recurrence from exfoliated tumour cell implantation. Then the spike of a transanally introduced intraluminal stapling device is passed through the middle of the transected rectal stump. The anvil of the stapling

instrument is secured around the proximal cut edge of the colon with a purse-string suture. Then the anvil is re-approximated to the spike, followed by closing and firing of the stapling instrument to achieve an anastomosis. A method of enabling the intraluminal circular stapling device to be introduced from the abdomen, rather than from the anus by performing a hand-sewn purse-string suture to the anorectal stump has been reported. This will be discussed in detail later, under "methods to preserve anal sphincter function".

Hand-sewn

Due the technical difficulties in manipulating tissue deep in the pelvis described above, the classical method of hand suturing with 'parachuting' stitches is seldom used in present times. Nonetheless, it is important that the surgeon who performs rectal surgery has the skills to perform a hand-sewn anastomosis on rare occasions when the stapling devices fail.

Pull-through hand-sewn coloanal

The circumstances for performing this type of anastomosis have already been discussed above under "introduction-intersphincteric dissection". In addition, this technique is an option in the obese patient with a narrow pelvis and in circumstances where an anastomosis needs to be salvaged after a stapling instrument mishap.

Stapled instrument malfunctioning

Aspects of salvaging the situation when the stapling instruments malfunction have already been described above, under "hand-sewn" and "pull-through hand-sewn coloanal anastomosis". It is not often possible to re-do the double cross stapled anastomosis, especially when the original rectal transection has been very distal. A very distal anastomosis can usually be accessed more easily for suture repair from the anus. In addition, the transanal route can be used to insert a purse-string for a repeated stapled anastomosis where the defect has been major. An appropriate defunctioning stoma would be essential.

Defunctioning stoma

It is well recognized that after total mesorectal excision, a distal colorectal/anal anastomosis at the level of the anorectal junction has a much higher risk of anastomotic dehiscence than more proximal colorectal anastomoses. Most of the time, these anastomotic problems are subclinical. There is a strong case for a defunctioning stoma to reduce the complications of an anastomotic leak even in routine circumstances. A defunctioning loop ileostomy is technically easier to fashion and to close, with less risk of damaging the marginal vessels than a colostomy. This has become the preferred technique by most surgeons in the West. However, a colostomy is preferable where the bowel preparation has been inadequate because residual faeces distal to a defunctioning stoma will continue to contaminate the anastomosis and hence will not reduce the complications of an anastomotic leak. For the same reasons, a colostomy would be preferable when the anastomosis is compromised or in places where

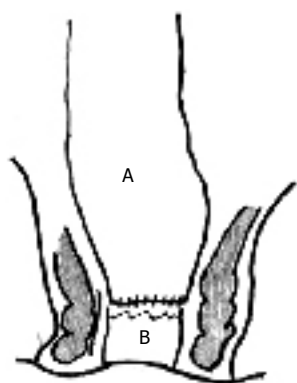


Figure 1 Straight coloanal anastomosis with colon (A) anastomosed directly to anorectum (B).

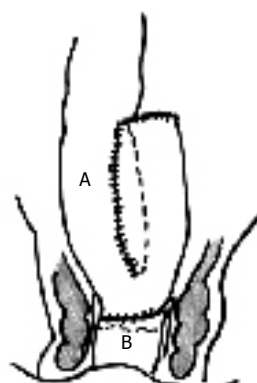


Figure 2 Colonic J-pouch-anal anastomosis with constructed colonic pouch (A) anastomosed to anorectum (B).

hot weather would cause excessive dehydration from an ileostomy. Traditionally, the defunctioning stoma is closed 12 wk later when the intraperitoneal adhesions would be more easily managed. This is provided that a contrast study confirms that the anastomosis is intact. In recent years, a case has been made for barrier agents against adhesions such as hyaluronate carboxymethylcellulose membranes (Seprafilm) to be placed around the stoma to enable earlier closure.

TYPES OF OPERATIONS

Using the above methods to re-anastomosis the bowel, 4 types of operations normally result i.e. 'straight' colorectal or coloanal anastomosis, colonic J-pouch anal anastomosis, coloplasty anastomosis & colonic side-to-end anastomosis.

Low anterior resection 'straight' anastomosis (Figure 1)

A straight anastomosis results from a direct end-to-end anastomosis of the colon to the anorectum. After segmental bowel resection (including right hemicolectomy, left hemicolectomy and anterior resection) for colorectal cancer, most patients (58%-78%) have a satisfactory 1 to 2 bowel movements/d^[8]. However after low anterior resection, up to one-third of patients have 3 or more bowel movements/d. At times, patients can be troubled with having up to 14 stools a day^[9,10]. Other patients may have defecation problems after anterior resection; excessive stool frequency or defecation problems under these circumstances has been termed as the "anterior resection syndrome".

Reasons for poor bowel function after 'straight' anastomosis

Multiple regression analysis on factors influencing postoperative bowel function showed that stool frequency at 1 year was independently predicted by the level of anastomosis and less importantly, the rectal sensation^[11]. This suggests that rectal reservoir function is important in controlling stool frequency. After ultra-low anterior resection with a straight colo-rectal/anal anastomosis, it has been shown that stool frequency depends upon the amount of rectum resected^[9,12-14]. When the level of anastomosis is less than 4-4.5 cm from the anal verge (ie. at approximately the anorectal junction), there is an increased risk of poor bowel function^[11,15]. After end-to-end coloanal (straight) anastomosis at the level of the

anorectal junction, the normally compliant rectum that has been removed is replaced by a less compliant segment of descending or sigmoid colon. The replacement colon is physiologically less suitable for storing/regulating feces^[11]. Not surprisingly, the results include excessive stool frequency and possibly fecal incontinence associated with the increased stool frequency. Another cause of post low anterior resection fecal incontinence is anal sphincter injuries, which occurs in up to 28 percent of patients following transanal insertion of a stapling instrument^[16]. This aspect will be discussed later (in "methods to preserve anal sphincter function").

Defecation problems may also occur in about 28% of patients after low anterior resection. This risk is similar to that after sigmoid colectomy (25%), which is substantially higher than the risk after more proximal bowel resections, such as right hemicolectomy (ranging from 4% to 15%)^[8]. This suggests that the sigmoid colon may have a major role in expelling and evacuating stools^[17], which would be consistent with the more muscular nature of this segment of large bowel. Resection causes discontinuity of colonic muscles and intrinsic nerves and, hence, disruption of coordinated colonic mass movement^[18]. In addition, division of the lateral ligaments of the rectum at ultra-low anterior resection may denervate the rectum and lead to significant constipation^[19].

Colonic J-pouch anal anastomosis (Figure 2)

The anterior resection syndrome symptoms have lead to various strategies to improve bowel function. To date, most of these strategies have focused on the proximal aspect of the anastomosis. This consists of various methods to better retain stool contents in the neorectum including the colonic J-pouch, coloplasty and colonic side-to-end anastomosis. The evolution of these techniques has been based on physiological considerations, which are hence discussed prior to the technique and results.

Physiology: When colonic J-pouches were first introduced, the aim was to maximize the neo-rectal compliance and volume by constructing a double barreled configuration with limbs sizes measuring up to 15 cm^[10,13,14,20-22]. Randomized controlled trials comparing this 15 cm colonic pouch technique to direct straight colo-rectal/anal anastomoses have confirmed improved stool frequency^[23-27]. Proctometrographic measurements have shown improved rectal volumes (i.e. increased neorectum

reservoir capacity) and rectal compliance (increased ability of the neorectum to retain high pressures)^[20,27]. However, these advantages came at the expense of severe evacuation problems^[27-30].

Meanwhile, a smaller 6 cm colonic J-pouch which is conveniently constructed from a single longitudinal firing of a linear cutting stapling instrument, was found to be effective in improving stool frequency, but with less rectal evacuation problems^[31,32]. This has since been confirmed by studies done in other centres^[26,33], and also in a randomized controlled trial comparing 6 cm with 9 cm colonic J-pouches^[34]. An interesting finding was that although there was improved function, no differences have been found in the rectal physiology (volume of initial sensation, maximum tolerable volume and compliance) measured at 1-year between small colonic J-pouch and straight coloanal anastomosis patients, in a randomized controlled trial^[32].

Compared to stationary anorectal manometric techniques, continuous ambulatory manometric monitoring has the advantage of monitoring pressure changes in the anus and rectum during prolonged periods and in a more physiologically normal environment. Using such techniques, patients with the smaller J-pouch were found to have a better tolerance to higher rectal pressures without increased stool frequencies, compared to straight anastomosis patients in a randomized prospective trial^[35]. The anorectal pressure gradient was also better preserved^[3], which had been described previously to be related to bowel frequency^[24,36]. Defecation in patients with colonic J-pouches may be related to large contraction waves (not corresponding to mass movement complexes) previously detected on ambulatory anorectal manometry, in patients with large colonic J-pouches^[36]. These large contraction waves were not observed after prolonged monitoring in patients with smaller 7 cm pouches, which might well explain the less severe rectal evacuation problems in these patients.

To elicit this further, a scintigraphy study protocol was designed to attempt to follow liquid faeces colonic transit with technetium ^{99m}Tc tin-colloid and solid faeces colonic transit with ^{131}I implanted microcapsules ingested at the same time^[37]. More technetium ^{99m}Tc tin-colloid was distributed into the liquid colonic contents at the distal colon at 24 h in 6 cm colonic J-pouch patients, than in those with straight coloanal anastomosis. This may be related to a backward stacking up effect from factors such as reversed peristalsis in the colonic J-pouch, accounting for the less frequent stools in those patients. The retention of ^{131}I in the solid stools were not different between colonic J-pouch and straight coloanal anastomosis patients, which may explain the rarity of severe evacuation problems (likely to involve solid stools) in patients with smaller 6 cm colonic J-pouches.

The barostat is a computerized pump that inflates a rectal balloon at controlled and reproducible rates of pressures, and volumes, providing a more accurate technique for assessing rectal physiology. The only study using barostat measurements on colonic J-pouches to date showed that there were no differences at 6 mo between 6 cm colonic J-pouch and straight coloanal anastomosis patients^[38]. At 2 years, there was a trend

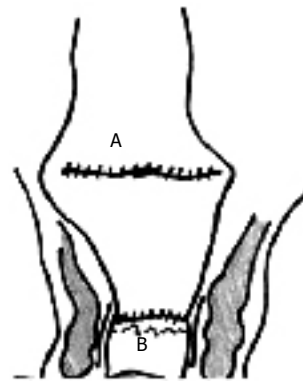


Figure 3 Coloplasty anastomosis with coloplasty in colon (A) anastomosed to anorectum (B).

for improved rectal sensation and maximum tolerable volume (as assessed with the phasic program) in both the types of patients. The phasic program assesses afferent sympathetic nerve function and hence these findings may be related to recovery of function in these nerves. Significant improvements in rectal compliance in straight coloanal anastomosis patients at 2 years had previously been documented in a cohort study, using traditional proctometrographic techniques^[23]. In addition, enlargements of the colonic J-pouch size have been measured radiologically over a 2 years period^[39]. All these changes may be responsible for the long term adaptation seen in patients after straight and colonic J-pouch colorectal/anal anastomoses.

Construction of 6 cm colonic J-pouch: Mobilization of the splenic flexure allows the descending colon to be used for the construction of the J-pouch. Very often this is necessitated by the sigmoid colon being badly affected by diverticulosis. Using a diseased sigmoid colon might compromise the pouch function and the anastomosis integrity. Where the sigmoid colon is healthy and of adequate length, it has been used instead of descending colon with no significant differences in stool frequency, incontinence, urgency, use of pads, need for antidiarrhoeal drugs, sensation of incomplete evacuation and anorectal physiologic results at 1-year follow-up^[40]. However, the descending colon has the advantage of being less muscular and more distensible than the sigmoid colon, which might improve the mid-term functional results^[38]. A comparison of results across studies which use descending and sigmoid colon at 2-year follow-up suggests that the descending colon adapts better^[38].

Coloplasty (Figure 3)

A coloplasty is designed to 'interrupt antegrade colonic peristalsis' and as an option when the pelvis is too narrow to permit a bulky colonic J-pouch anal anastomosis^[41]. It is similar to a pyloroplasty or stricturoplasty, and had initially been tried out in pigs^[42,43] prior to testing out in patients^[44,45].

Construction of coloplasty: A 7 cm longitudinal incision is made between the taenia along the anti-mesenteric side of the descending colon, starting 4 cm above the distal cut end. The incision is closed transversely with a continuous single layer of seromuscular absorbable suture. The coloplasty 'pouch' is then anastomosed to the stapled anorectal stump by a double cross stapling technique with

the coloplasty facing anteriorly.

Side-to-end anastomosis (Figure 4)

The side-to-end anastomosis that was first described by Baker JW in 1950^[45], has been revisited recently as another option to improve postoperative bowel function. There are 2 variations: (1) anastomosis performed by introducing the intraluminal stapler from the anus or (2) anastomosis done entirely from the abdomen alone. Using the former variation, Machado M *et al*^[46] performed a randomized controlled trial which showed that the side-to-end anastomosis was functionally comparable to the 8 cm colonic J-pouch. The only difference was better neorectal evacuation in < 15 min in the colonic J-pouch group, at 6 mo. The second variation of the side-to-end anastomosis hinges upon the concept of preserving anal sphincter function per se (as compared to improving proximal bowel motility or reservoir function), and hence that is discussed in further detail.

Methods to preserve anal sphincter function: After low anterior resection, faecal continence may be compromised in 13%-80% of patients^[5,9,10]. It is known that some damage to the anal sphincters can occur after low anterior resection^[35,47,48]. Anal pressures are significantly impaired and the rectosphincteric inhibitory reflex recovered only in a few patients at 2 years after low anterior resection, regardless of any reservoir construction^[38]. The rectosphincteric inhibitory reflex requires an intact reflex pathway based upon the anal sphincter muscles and intrinsic rectal innervation. Ambulatory anorectal manometric studies have shown that after low anterior resection, patients who complained of soiling while passing flatus had lower minimal anal pressures^[35]. The latter is very likely related to injury to the internal anal sphincter during transanal stapling instrument insertion^[24,35,36].

Horgan *et al*^[49] monitored the anal pressures of patients on the operating table undergoing anterior resections. They found that the anal pressures were maintained at the division of the inferior mesenteric artery, full mobilization of the rectum and mesorectum, and anal transection. The anal pressures only decreased significantly after transanal introduction of the intraluminal circular stapler, suggesting direct injury during anal insertion of the instrument. Molloy *et al*^[50], found that anal resting pressures in dogs was significantly lower after transanal introduction of intraluminal circular stapler than handsewn colorectal anastomoses. However, it was likely that nerve injury during rectal mobilization was also important in dogs, because there was significant impairment of anal pressures after both types of anastomoses. A randomized controlled trial showed that direct injuries to the internal anal sphincter occurred after transanal insertion of the stapler but not with the biofragmentable anastomotic ring (where the anastomosis was performed entirely intra-abdominally) after high anterior resection^[51]. The nature of the injury was documented with endoanal ultrasound in a follow-up randomized controlled clinical study to be the result of anal sphincter injuries^[16]. In order to avoid transanal introduction of the intraluminal stapling device, the second variation of the side-to-end anastomosis was devised to

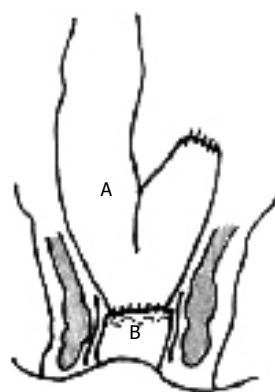


Figure 4 Side-to-end anastomosis with side of colon (A) anastomosed to end of anorectum (B).

enable a stapled anastomosis performed entirely from the abdomen. Huber FT *et al*^[52] reported on a randomized controlled trial which compared this variation of side-to-end anastomosis (with 3-4 cm blind end) with the 6 cm colonic J-pouch.

COLONIC J-POUCH-ANAL ANASTOMOSIS

Complications

The better-vascularized end-to-side anastomosis with a J-pouch has been shown to reduce the risks of anastomotic dehiscence, compared to the straight coloanal/rectal anastomosis^[23]. This will be discussed in detail in the section on 'coloplasty'.

Function

Early function (1 year): Patients with a small 6 cm colonic J-pouch-anal anastomosis have a median of 3 bowel movements a day compared with a median of 6 a day for patients with straight anastomoses, at 1 year after surgery^[31,32]. Urgency to defecate is less troubling in colonic J-pouch patients. However, a frequent sensation of incomplete neorectal evacuation was more common after the small colonic J-pouch-anal anastomosis, but most of these patients do not require suppositories, laxatives or enemas to evacuate.

Late function (2 years): At 2 years of follow-up, patients with 6 cm colonic J-pouch and those with straight coloanal anastomoses have similar bowel frequency at about 1 bowel movement a day^[38]. The need for anti-diarrhoeal medications is minimal in both groups. In the only other randomized controlled trial with long-term follow-up, Lazorthes *et al*^[24] reported no improvements in their straight coloanal anastomosis patients at 2 years. This may well be due to using the sigmoid colon for some of their coloanal anastomoses. As the sigmoid colon is less distensible, it is less likely to adapt successfully in the long-term as a storage reservoir for faeces. This would account for the no difference in bowel function between colonic pouch and straight anastomosis patients, reported in the other available data from cohort studies^[23,53]. Dehni *et al*^[54] reported that stool frequency remained superior in the pouch patients after 5 years but conceded that they had difficulty assessing stool frequency because of significant stool fragmentation in their patients.

Bowel continence has been reported by some to be

better in colonic J-pouch patients than in those with straight coloanal anastomoses^[11,23,25,26]. However, the differences are often minor, like less likelihood of soiling with passing flatus^[38]. At 2 years, studies to date have confirmed no differences in continence with either anastomoses^[23,38,41,43]. At this stage, unless there is excessive stool frequency, it is likely that significant faecal incontinence is related more to anal sphincter injuries than to neorectal reservoir function (see below).

Bowel evacuation is improved at 2 years with straight coloanal anastomosis patients, but major evacuation problems remain minimal with colonic J-pouch patients^[38]. A randomized controlled trial comparing the function of 6 cm and 9 cm colonic J-pouches at 2 years showed that fewer 6 cm pouch patients required laxatives and enemas for severe constipation^[34]. Stool fragmentation/clustering has been defined as multiple evacuations over a 1-2 h period associated with persistent sensation of rectal fullness. More straight anastomosis patients had persistent long-term stool fragmentation^[53,54]. As this phenomenon has not been confirmed in the only other large randomized controlled trial to date, these findings well may be related to cultural and dietary factors^[38]. The results to date suggest that the small 6-7 cm colonic J-pouch-anal/rectal anastomosis is the procedure of choice because of early improved bowel function and less risk of anastomotic complications.

COLOPLASTY ANAL ANASTOMOSIS

Complications

A randomized controlled trial comparing colonic J-pouch with coloplasty 'pouch'-anal anastomosis showed significantly more anastomotic leaks in the latter (15.9%)^[41]. Seven percent were clinical and 9% were radiologic, found at routine barium enema prior to ileostomy closure. All the leaks were at the anterior of the coloanal anastomoses, below the site of the coloplasty. Anastomotic leaks were not significantly associated with postoperative chemotherapy or radiotherapy ($P = 0.417$). A 5% incidence of clinical anastomotic leak had been reported in the only other series on coloplasty pouch patients (*nonrandomized*) to date^[45]. The lower incidence of anastomotic leak after J-pouch may be due to better proximal anastomotic blood supply, as shown by the laser Doppler technique. This better blood supply at the critical anastomotic site was related to the J-pouch being anastomosed side-to-end to the anal canal, compared with the straight coloanal, which is an end-to-end anastomosis. Hence, higher leak rates after coloplasty may be due essentially to the coloanal anastomosis being made end-to-end, as in the straight coloanal anastomoses. The reported coloplasty leak rates were comparable to the straight anastomosis leak rates. An additional possibility to consider would be some compromise of blood supply to the colonic anastomotic end as a result of the coloplasty. This may account for all the clinical and radiologic leaks occurring anteriorly at the coloanal anastomosis, just distal to the coloplasty.

Function

The differences between the early functional results of

the small colonic J-pouch and coloplasty techniques are subtle^[41]. J-pouch patients had significantly less stool fragmentation, which required returning to the toilet at least once within 15 min of evacuation. However, both groups of patients reported increased stool fragmentation at 1 year. It is known that patients may have difficulty in differentiating stool fragmentation from increased bowel frequency. No differences were found with other stool evacuation problems. However, coloplasty patients had significantly better stool deferment time and less nocturnal liquid stool leakage. No differences were found with other stool incontinence symptoms and with continence scoring. The only other published study on the coloplasty technique compared 20 patients with historical controls consisting of 16 J-pouch and 17 straight coloanal anastomoses^[45]. It confirmed no significant differences between coloplasty and colonic J-pouch patients when stool frequency, use of antidiarrheal medication, and continence were compared. Patients' perceptions as measured by the Fecal Incontinence Quality of Life scale also showed no difference between the small colonic J-pouch and coloplasty techniques^[41].

Physiology

Anorectal manometric findings did not show any significant differences in the function of small colonic J-pouch and coloplasty patients^[41]. Colonic pouch reservoir function, as measured by the rectal volume of initial sensation, maximum tolerable volume, and compliance, was not different between the groups. This was consistent with the findings of Mantyh *et al*^[45], mentioned above. Z'graggen *et al*^[42] also found no differences in maximum tolerable volume and compliance between J-pouch and coloplasty in their experimental surgery on 15 pigs. Although experimental surgery construction of a coloplasty may provide a 40% increase in volume^[42], it is more than likely that in the clinical situation motility factors such as disruption of colonic propulsion as a result of the coloplasty on the antimesenteric surface may be more important^[44].

At this time because of higher risks of anastomotic complications, coloplasty cannot be recommended except for special circumstances when a bulky J-pouch cannot be brought through a narrow pelvis for anastomosis to the anorectal junction. Nonetheless, further studies with variations in coloplasty design and longer follow-up may reveal other advantages in the technique. However, due caution, including the use of defunctioning stoma and careful postoperative observation, should be exercised.

SIDE-TO-END COLOANAL ANASTOMOSIS

Complications

As both the colonic J-pouch and the side-to-end anastomoses are essentially side-to-end anastomoses, the vascularity and the related complications would be expected to be similar. This has proven to be the case in studies. A unique complication of the side-to-end anastomosis technique is bowel obstruction related to inadvertent inclusion of the side-wall of the opposite limb into the anastomosis. Unlike the colonic J-pouch, the side-

to-end anastomosis has a narrow lumen which makes inclusion of the side-wall a potential risk.

Function

The colonic J-pouch group was reported to have better stool frequency at 3 mo, but the functional results equalized in both groups at 6 mo^[52]. It was interesting that despite the different methods of introducing the intraluminal stapling instrument, there were no differences in postoperative continence and anal manometric findings. It is conceivable that a similar mechanism to 'reverse peristalsis' in the small colonic J-pouch would occur with a 3 to 5 cm blind limb side-to-end anastomosis. With some distension over time, the final anatomic configuration of the latter may well conform to that of the 5 cm colonic J-pouch. However with regard to incontinence related to anal sphincter injuries, there were no differences between the transabdominal and transanal introduction of the stapling device^[52]. When total mesorectal excision has been properly performed, the distal mesorectum would have been lifted off the pelvic floor. The rectum and the mesorectum would be transected at the level of the anorectal junction. Application of a purse-string suture from the abdomen would be a difficult exercise, particularly in Western patients who are usually more heavily built. In particular, balancing the conical or narrow disc shaped stapler anvil in the anal canal without having it squeezed out by the anal muscles whilst securing the purse-string suture at the anorectal junction at the same time is no mean feat.

MANAGEMENT OF 'ANTERIOR RESECTION' SYNDROME

Conservative

Traditionally, management of poor bowel function has been managed expectantly having excluded other causes particularly tumour recurrence and pelvic sepsis. It is now known the colonic adaptation can take up to 18 mo to occur after ultra-low anterior resection with total mesorectal excision^[38]. The patient is advised to take adequate soluble fibre in the diet and to avoid foods which aggravate the bowel dysfunction. Those with increased stool frequency may be prescribed diphenoxylate, codeine and/or bile salt binding agents to help control the symptoms. Patients with rectal evacuation problems may be prescribed regular laxatives and enemas. On rare occasions, patients fail to respond to conservative treatment with persisting debilitating bowel function lasting beyond 18 mo. Under such circumstances, anorectal biofeedback therapy and/or postanal sphincter repair may need to be considered. Resort to a stoma would be only needed in very exceptional circumstances only, where patients are managed in a specialist colorectal surgical centre.

Anorectal biofeedback

Biofeedback has been shown to be effective in treating certain types of faecal incontinence^[55]. This is a specific form of behaviour modification that aims to control

bodily function. Biofeedback has been reported to be successful in managing patients who have stool frequency and/or incontinent problems after anterior resection^[56]. At a mean follow-up of 10.6 mo, 90% success with no regressions or complications was found. Anorectal physiologic tests done before and after biofeedback show minimal increase in anal pressures. It is possible that biofeedback works by improving the anal sphincteric coordination, rectal sensation, rectal liquid retention and/or anal canal sensation. Although patient-biofeedback therapist relationship may be vital, none of the patients received any formal psychiatric counseling. Biofeedback has also been reported to be 90% successful in managing intractable constipation following low anterior resection^[57]. Intractable constipation after low anterior resection is likely to result from resection of the sigmoid colon, which is the main propulsive organ of the large intestine^[17]. Again, the results of pre and post-biofeedback anorectal physiologic tests are inconclusive, suggesting that similar factors outlined previously may play an important role in bringing about the positive changes in bowel function.

Postanal sphincter repair

Treatment options are limited for persistent intractable excessive stool frequency and incontinence after low anterior resection for rectal cancer. Fortunately, this is quite rare, but such patients treated successfully by postanal sphincter repair have been reported^[58]. In such cases, it is essential that anorectal physiologic tests and endoanal ultrasound findings are consistent with internal anal sphincter injuries, which are known to occur with transanal insertion of stapling instruments. After postanal sphincter repair, stool frequency was reported reduced from a mean 5.7 to 1.7 stools per day^[58]. Fecal incontinence requiring pads in all patients was improved to full continence in 67% and minor incontinence for flatus in 33%. Continence score improved from a mean 13.7 to 1.3. Mean follow-up was 3.2 years. With the recent advent of bulking agents implanted intersphincterically by injection, another option for managing internal sphincter injuries in patients after low anterior resection is now available. Clinical studies are awaited.

LAPAROSCOPIC ULTRA-LOW ANTERIOR RESECTION

Laparoscopic colonic cancer surgery has been proven to be at least as safe and effective as traditional open surgery. However, data is still sparse with regard to the laparoscopic management of rectal cancer. It is now technically possible to perform the mobilization of the left colon and total mesorectal excision by a laparoscopic technique. The anorectal junction can be stapled and transected with an endoscopic linear cutter stapler. The specimen can then be extracted through a plastic drape protected 4-5 cm muscle splitting transverse incision, which can be used eventually for the temporary defunctioning stoma. A colonic J-pouch or coloplasty can be performed extracorporeally and pneumoperitoneum reconstituted to perform an intracorporeal end-to-end double cross

stapled anastomosis, with the intraluminal stapling device introduced transanally. Further data and refinement of the technique is awaited.

In conclusion, at present, the small colonic J-pouch-anal anastomosis is the most widely accepted method of restoring colonic anal continuity after a total mesorectal excision. A straight colorectal anastomosis is preferred where the anastomosis is more than 4–6 cm above the anal verge. In these circumstances, there is adequate residual rectum to provide the necessary rectal reservoir capacity. Besides, performing a colonic J-pouch-rectal anastomosis at this proximal level is likely to result in rectal evacuation problems. Where a coloanal anastomosis at the anorectal junction needs to be considered in a heavily built patient with a narrow pelvis, a coloplasty can be considered. The other methods of restoring bowel continuity are best kept for special circumstances such as stapling gun misfiring, where their unique technical features will help salvage the operation, where others are not possible. A laparoscopic technique is likely to be the method of choice in the near future.

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Hepatic progenitor cells in human liver tumor development

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Abstract

In recent years, the results of several studies suggest that human liver tumors can be derived from hepatic progenitor cells rather than from mature cell types. The available data indeed strongly suggest that most combined hepatocellular-cholangiocarcinomas arise from hepatic progenitor cells that retained their potential to differentiate into the hepatocytic and biliary lineages. Hepatic progenitor cells could also be the basis for some hepatocellular carcinomas and hepatocellular adenomas, although it is very difficult to determine the origin of an individual hepatocellular carcinoma. There is currently not enough data to make statements regarding a hepatic progenitor cell origin of cholangiocarcinoma. The presence of hepatic progenitor cell markers and the presence and extent of the cholangiocellular component are factors that are related to the prognosis of hepatocellular carcinomas and combined hepatocellular-cholangiocarcinomas, respectively.

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Key words: Hepatic progenitor cell; Hepatocellular carcinoma; Liver tumor

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INTRODUCTION

The traditional view that adult human liver tumors arise from mature cell types has been challenged in recent decades. The results of several studies suggest that some types of human liver tumors can be derived from hepatic progenitor cells (HPCs). In this review, the evidence for a HPC origin of several types of liver tumors and the pos-

sible clinical implications are discussed.

DEFINITION OF HEPATIC PROGENITOR CELLS IN HUMAN LIVER

HPCs are small epithelial cells with an oval nucleus, scant cytoplasm and location in the bile ductules and canals of Hering^[1]. HPCs can differentiate towards the biliary and hepatocytic lineages. Differentiation towards the biliary lineage occurs *via* formation of reactive bile ductules, which are anastomosing ductules lined by immature biliary cells with a relatively large and oval nucleus surrounded by a small rim of cytoplasm. Hepatocytic differentiation leads to the formation of intermediate hepatocyte-like cells, which are defined as polygonal cells with a size intermediate between that of HPCs and hepatocytes^[1]. In most liver diseases, hepatic progenitor cells are “activated”, which means that they proliferate and differentiate towards the hepatocytic and/or biliary lineages. The extent of activation is correlated with disease severity^[2-4].

HPCs and their immediate biliary and hepatocytic progeny not only have a distinct morphology, but they also express several markers, with many also present in bile duct epithelial cells^[1,5]. Immunohistochemistry using antibodies against these markers facilitates the detection of HPCs. The most commonly used markers are cytokeratin (CK) 19 and CK7^[1,6].

HEPATOCELLULAR CARCINOMA AND HEPATOCELLULAR ADENOMA

Evidence for a hepatic progenitor cell origin of hepatocellular carcinoma and adenoma

The proposal that a human hepatocellular carcinoma (HCC) does not necessarily arise from a mature hepatocyte, but could have a HPC origin, has classically been based on three different types of observations. Each of them, however, gives only indirect evidence that can be disputed.

Firstly, it has been shown that HPCs are the cells of origin of HCC in some animal models of hepatocarcinogenesis^[7,8], which has led to the suggestion that this might also be the case in humans. However, in other animal models, the HCCs arise from mature hepatocytes and not from HPCs or reactive bile ductular cells^[9,10]. Since it is currently insufficiently clear which of these animal models accurately mimics human hepatocarcinogenesis^[11,12], one should be careful about extrapolating data regarding the HPC origin of HCC in animal models to the human situation.

Secondly, liver diseases that are characterized by the

presence of carcinogens and development of dysplastic lesions also show HPC activation^[1]. Therefore, the suggestion has been made that HPCs form a “target population” for carcinogens^[3,13], but this is only a theoretical possibility not supported by experimental data.

Thirdly, several studies have shown that a considerable proportion of HCCs express one or more HPC markers that are not present in normal, mature hepatocytes^[14-18]. Due to the fact that most HPC markers are also expressed in the biliary lineage, the term “biliary marker” has been used in some of these studies^[16,17]. The “maturation arrest” hypothesis^[19] states that genetic alterations occurring in a HPC, or its immediate progeny, cause aberrant proliferation and prevent its normal differentiation (Figure 1). Further accumulation of genetic alterations eventually leads to malignant transformation of these incompletely differentiated cells. The resulting HCC expresses HPC markers as evidence of its origin. However, expression of HPC markers can also be interpreted in the setting of the “dedifferentiation” hypothesis^[19], which suggests that the expression of HPC markers is acquired during tumor progression as a consequence of accumulating mutations (Figure 1). For example, experiments in which human HCC cell lines were transplanted into nude mice have nicely shown that the expression of the HPC marker, CK19, steadily increased when the tumors became increasingly aggressive and metastasized to the lungs^[20]. Thus, the expression of CK19 in a HCC does not necessarily mean that the tumor has a HPC origin, but it can also be a mutation-induced, acquired expression associated with tumor progression. Both possibilities are not mutually exclusive.

To further address this issue, precursor lesions of HCCs have been investigated for the presence of HPCs and their immediate hepatocytic progeny. It was found that HPCs and intermediate hepatocyte-like cells were present in 50% of small cell dysplastic foci and hepatocellular adenomas^[21,22]. Since both types of these lesions are precursors of HCC^[12], this supports the idea that at least some HCCs are HPC-derived. Indeed, if the expression of HPC markers in HCCs would always be acquired during tumoral progression, these precursor lesions would not contain HPCs and their immediate hepatocytic progeny (Figure 1). Furthermore, HPCs and intermediate hepatocyte-like cells in hepatocellular adenomas were present in a “starry sky” pattern on a background of mature hepatocytes (Figure 2)^[22]. This picture is in agreement with the “cancer stem cell” theory: stem cells that have undergone transformation retain their renewal properties and nourish the tumor by their continuous proliferation and differentiation^[23]. Hepatocellular adenomas with HPCs and intermediate hepatocyte-like cells did not form a specific clinicopathological subtype^[22].

Although these findings further support a HPC origin for some human HCCs, they certainly do not give definite proof. Moreover, these are findings and hypotheses that apply to HCC in general. For an individual HCC that expresses a HPC marker, it remains impossible to determine whether this marker reflects the cellular origin and/or is caused by tumor progression. This can only be elucidated by determining whether the HCC contains cells that are ultrastructurally identical to HPCs in non-tumoral liver. Until now, this type of investigation has only been done for

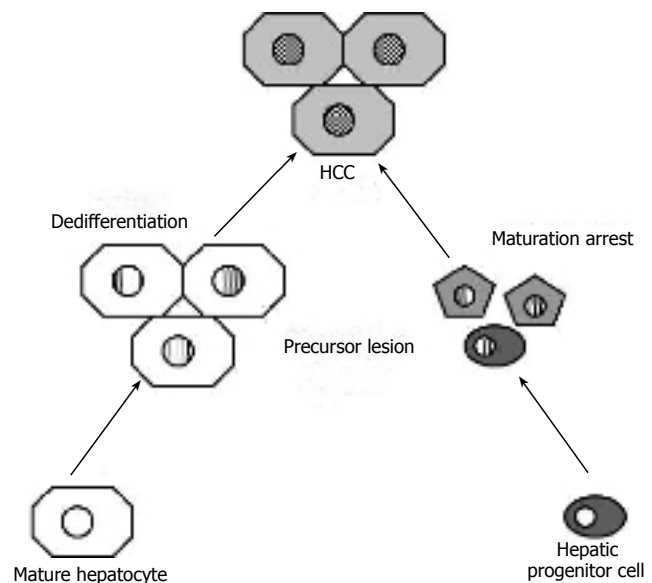


Figure 1 Schematic representation of the “maturation arrest” hypothesis and the “dedifferentiation” hypothesis applied to hepatocellular carcinoma and its precursor lesions. According to the “maturation arrest” hypothesis, genetic alterations (grid in nuclei) occurring in hepatic progenitor cells causes proliferation and incomplete differentiation of these cells, eventually leading to hepatocellular carcinomas that express hepatic progenitor cell markers (grayscale) as an evidence of their cellular origin. In this scenario, precursor lesions contain hepatic progenitor cells and intermediate cells (the small polygonal cells). The “dedifferentiation” hypothesis states that the expression of hepatic progenitor cell markers in hepatocellular carcinoma is merely a result of tumor progression and consequently the precursor lesions should only consist of mature hepatocytes.

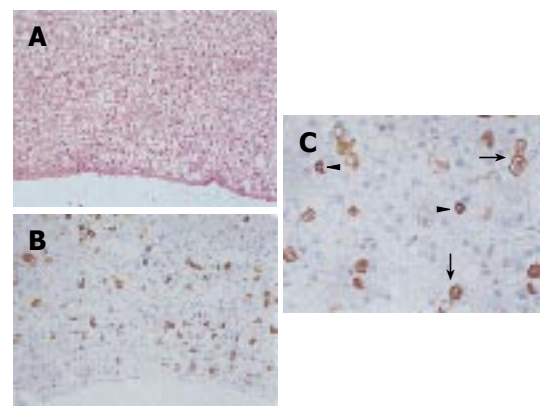


Figure 2 Hematoxylin-eosin stained section of a hepatocellular carcinoma, which suggests that the tumor consists of mature hepatocytes (A). However, the staining for the hepatic progenitor cell marker cytokeratin 7 (B and C) shows the presence of hepatic progenitor cells (arrowheads) and intermediate hepatocyte-like cells (arrows) distributed in a “starry sky” pattern against a background of mature hepatocytes. Original magnification: x 100 (A and B) and x 200 (C).

some hepatocellular adenomas and a very small number of HCCs^[22,24].

Prognostic value of the expression of hepatic progenitor cell markers in hepatocellular carcinoma

From a purely clinical viewpoint, the reason why some HCCs express HPC markers is not important. However, the fact that several studies have shown that HCCs expressing HPC markers have a worse prognosis than HPC

marker-negative HCCs might be potentially clinically relevant^[14-17]. All but one^[14] of these studies also reported that HPC marker-positive HCCs were less differentiated than marker-negative HCCs. Multivariate analysis was performed in only one of these studies and expression of the HPC marker CK19 was an independent predictor of postoperative recurrence^[15]. This finding needs to be confirmed before one can consider using CK19-expression for prognostication instead of more strongly established independent prognostic markers, such as the immunohistochemical expression of the cell proliferation marker Ki-67^[25-27].

In a recent study, unsupervised clustering of microarray data from human HCCs and rat hepatoblasts led to the identification of a subgroup of HCCs that resembled rat hepatoblasts, designated “hepatoblast (HB) subtype” of HCC^[11]. This HB subtype of HCCs was characterized by the high expression of several HPC markers, such as CK7 and CK19. However, the expression of other known HPC markers, such as alpha-fetoprotein and ABCB1^[28,29], was not different and was even lower, respectively, in HB subtype HCCs when compared to the “hepatocyte subtype” of HCCs. Therefore, the exact relationship between HPCs and this HB subtype of HCCs remains unclear. Remarkably, HB subtype HCCs consisted of tumors associated with a very poor prognosis and the HB signature had a very strong and independent prognostic power. Unfortunately, direct application in clinical practice will not be possible because microarrays can not be performed in every hospital and, more importantly, the method relies on unsupervised clustering. This means that adding new HCCs to the group most likely will alter the dendrogram and hence also the subtype of several of the already included HCCs. This effect has already been observed in breast cancer^[30].

COMBINED HEPATOCELLULAR-CHOLANGIOCARCINOMA

Three subtypes of combined hepatocellular-cholangiocarcinoma (CHC) can be distinguished. The first type consists of the so-called “collision tumors” and “double cancers” in which the HCC and the cholangiocarcinoma components are either completely separated or sharply demarcated from each other, indicating that these tumors arise from the coincidental occurrence (and collision) of a HCC and a cholangiocarcinoma^[31,32]. The possible HPC origin of the independent components are discussed in the sections on HCC and cholangiocarcinoma.

The second type of CHC represents tumors containing unequivocal (i.e. recognizable on a hematoxylin-eosin-stained section) hepatocellular and cholangiocellular components that are intimately admixed^[33,34]. In almost all cases, these tumors also show a variable amount of so-called “transitional areas” that consist of immature-appearing cells that have morphological and immunohistochemical features of both hepatocytes and cholangiocytes^[32,33,35]. The cells in the transitional area sometimes resemble HPCs and reactive bile ductules in the non-tumoral liver^[33,35-37]. Overall, this picture strongly suggests that the origin of these

tumors lies in a HPC that has undergone malignant transformation while differentiating in both the hepatocytic and biliary lineages. It is not completely excludable that the cholangiocellular components are directly derived from the hepatocellular components or *vice versa*, but this can not account for the presence of tumor cells with morphological, immunohistochemical and architectural features reminiscent of HPCs and reactive bile ductules.

CHCs that consist (almost) completely of such transitional areas and contain only very limited or no unequivocal hepatocellular and cholangiocellular components have been designated as “primary liver carcinoma of intermediate (hepatocyte-cholangiocyte) phenotype” and they are thus also likely to have a HPC origin^[37,38]. However, only a few cases of this type of tumor have been described, so it is currently not clear whether this third CHC subtype has clinical and prognostic features that are distinct from the second subtype of CHCs.

In contrast, there exist several rather large studies (i.e. at least 20 patients) on the clinicopathological features of the second type of CHCs, including a comparison with HCC and cholangiocarcinoma^[35,39-42]. Although the results of these studies are conflicting regarding the associations with cirrhosis and hepatitis B or C infection, there is a general consensus that these CHCs are almost always invasive and have a prognosis that lies in between that of HCC and cholangiocarcinoma. A recent study has nicely revealed that CHCs with an extensive cholangiocellular component are more frequently invasive and metastatic to lymph nodes and have a worse prognosis than those with a limited cholangiocellular component^[35]. So, it is the extent of differentiation towards the biliary lineage rather than the HPC origin as such that determines the specific prognostic features of this type of tumor.

CHOLANGIOCARCINOMA

The issue of whether some cholangiocarcinomas could arise from HPCs rather than from mature cholangiocytes has not yet been investigated in detail. It has been shown that cholangiocarcinoma can originate from HPCs in some animal models^[43,44] and a considerable proportion of human cholangiocarcinomas express the neural cell adhesion molecule, which is a marker for reactive bile ductules not present in mature cholangiocytes^[45]. However, more studies are needed before statements regarding the HPC origin of cholangiocarcinoma can be made.

CONCLUSION

The available data strongly suggest that most CHCs arise from HPCs. Furthermore, HPCs could also be the basis of some HCCs and hepatocellular adenomas, although it is very difficult to determine the origin of a specific, individual HCC. There is currently not enough data to make statements regarding a HPC origin of cholangiocarcinoma. The presence of HPC markers and the presence and extent of the cholangiocellular component are factors that are related to the prognosis of HCCs and CHCs, respectively.

ACKNOWLEDGMENTS

Figure 2 is reprinted with permission from: Libbrecht *et al.* Hepatic progenitor cells in hepatocellular adenomas. *Am J Surg Pathol* 25: 1388.

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REVIEW

Technological insights: Combined impedance manometry for esophageal motility testing-current results and further implications

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Abstract

This review focuses on current aspects of the novel technology of combined impedance manometry for esophageal motility testing. It presents methodological features, summarizes current results and discusses implications for further research. The combined technique assesses simultaneously bolus transport and associated peristalsis, thus allowing detailed analysis of the relationships between bolus transit and esophageal motility. Recent studies demonstrate that combined impedance manometry provides important additional information about esophageal motility as compared to conventional manometry: (1) monitoring of bolus transport patterns, (2) calculation of bolus transit parameters, (3) evaluation of bolus clearance, (4) monitoring of swallow associated events such as air movement and reflux, and (5) investigation of the relationships between bolus transit and LES relaxation. Studies with healthy subjects have identified several useful parameters for comprehensive assessment of esophageal function. These parameters were found to be pathological in patients with classical achalasia, mild GERD, and ineffective esophageal motility. The technology of combined impedance manometry provides an important new tool for esophageal function testing, advancing both clinical and basic research. However, several important issues remain to be standardized to make the technique suitable for widely clinical use.

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Key words: Combined impedance manometry; Esophageal function testing; Review

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INTRODUCTION

The basic function of the esophagus is the transport of the bolus from the pharynx into the stomach. Esophageal peristalsis is based on propulsive mechanisms along the axis of the organ, generated by a latency gradient that is modulated by the inhibitory neurotransmitter nitric oxide^[1-3]. Much is known about the physiological and pathological phenomena of peristalsis based on intraluminal manometry^[4], and up to now, this technique is the standard method to study esophageal motility^[5-7]. But until recently, the relationships between peristalsis, intrabolus forces and bolus transport could be assessed by radiological contrast studies only^[8-10]. For this purpose, other technologies have emerged, such as ultrafast computed tomography^[11,12], intraluminal high frequency esophageal ultrasonography^[13], and topographical esophageal manometric methods^[14]. However, these techniques are expensive, require specific technical support and personal expertise, and do not clarify some details of bolus transport along the esophagus.

In 1991, Silny described a new catheter-related procedure for high-resolution measurements of gastrointestinal motility and bolus transport based on intraluminal measurements of electrical impedance^[15]. Subsequently, this technique was validated by means of manometry and videofluoroscopy studies^[16-18] and it was applied for studying of intestinal chyme transport^[19,20], for monitoring of reflux^[21-23], and for evaluation of esophageal bolus transport^[24].

However, the impedance technique alone also has some limitations. The contraction amplitude, an important parameter in predicting organ function, cannot be determined^[25]. Impedance measurements without manometry can underestimate some aspects of the relationships between esophageal wall movement and bolus motion, especially in patients with dysphagia or chest pain. In order to cope with these difficulties, a second generation of impedance catheters was developed^[26]. The catheter integrates impedance monitoring and manometry

in a single device. Thus, both tests can be performed simultaneously and the relationships between the dynamics of bolus transport and wall motion can be analysed well, while the quality of recording is maintained.

Recently combined impedance manometry has been increasingly applied for esophageal motility testing^[27-36]. This report summarizes current results and discusses future prospects of this novel technique.

SCIENTIFIC BASICS

The method is based on the esophageal intraluminal measurement of electrical impedance and pressure between a number of arranged impedance electrodes and pressure sensors during a bolus passage using an intraluminal probe (Figure 1).

The intraluminal electrical impedance is inversely proportional to the electrical conductivity of the luminal contents and the cross-sectional area (Figure 1). Compared to the muscular wall, air has a lower electrical conductivity and yields increased impedance. In contrast, saliva or nutrients show a higher conductivity and therefore cause an impedance drop at the corresponding measurement segments. On the other hand, luminal dilatation results in an impedance drop, whereas luminal narrowing causes an increase in impedance^[15].

The bolus passage along each measured segment allows the delineation of the typical tracing of impedance, which includes a maximum of five phases (Figure 2, upper panel): (1) phase 1 is the resting stage of the organ; (2) phase 2 represents the facultative arrival and passage of an air volume ahead of the bolus; (3) phase 3 is associated with the arrival and the passage of a bolus. The initial rapid fall of impedance is associated with the arrival of the bolus front as bolus entry (F-Point). During the subsequent nearly plateau phase the bolus is mainly located within the measuring segment; the minimum impedance during this phase represents the bolus body (B-Point); (4) during phase 4 the bolus leaves the measuring segment as bolus exit due to wall contraction with facultative lumen occlusion, which can be represented by the maximum impedance (C-Point); (5) phase 5 is the transitory stage to resting stage. This characteristic impedance wave form may change in the case of absence of air in front of the bolus or absence of a lumen-occluding contraction wave (Figure 2, upper panel). For visualization of the maximum and minimum impedance values an individual scaling (Figure 2, lower panel, left side) can be used instead of the standard scaling (Figure 2, lower panel, right side).

The F-Point, B-Point and C-Point can be determined by computer assistance according to the presumed definitions, as shown in Figure 3, left panel. Alternatively, bolus entry and exit have been defined as follows^[33,34]: Bolus entry is considered to occur at the 50% point between impedance baseline and impedance nadir during bolus passage, and bolus exit is determined as 50% point on the impedance recovery curve, as shown in Figure 3, right panel.

EQUIPMENT AND TECHNOLOGY

There are two prototypes of combined impedance

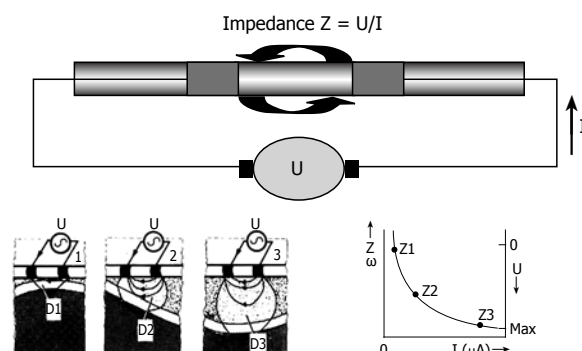


Figure 1 Upper panel: Principles of intraluminal impedance manometry: the electrical impedance (Z) of a electric field between 2 electrodes is the ratio between applied voltage (U) and resulting current (I). Lower panel: Impedance is non-linearly inversely dependent on bolus diameter and electrical conductivity of luminal content.

manometry catheters available (Figure 4)

(1) 15-channel esophageal function testing catheter^[26-30]: the impedance-manometry catheter is a polyvinyl catheter with an external diameter of 2.5 mm. The catheter consists of 11 impedance segments (each 2 cm long) and 4 semiconductor solid-state pressure transducers to register the manometry tracings. The solid-state pressure transducers are located at the junction between the impedance channels 1-2, 4-5, 7-8, and 10-11, with an intertransducer distance of 6 cm (Figure 4, left panel). (2) 9-channel esophageal function testing catheter^[31-35]: it incorporates five pressure (two circumferential and three unidirectional) sensors and four impedance-measuring segments. The two circumferential solid-state pressure sensors are located at 5 cm and 10 cm from the tip and three unidirectional pressure sensors at 15, 20, and 25 cm. Impedance measuring-segments consist of pairs of metal rings placed 2 cm apart, centered at 10, 15, 20 and 25 cm from the tip, thus spanning the four proximal pressures transducers (Figure 4, right panel).

The first system^[26-30] displays a cascade configuration of the impedance measuring segments similar to the systems used in previous impedance studies for the study of bolus transport^[19,20,24] and for monitoring of reflux^[21,22]. The pressure transducers also serve as impedance electrodes and are located exactly at the junction between 2 adjacent impedance segments (the end of one segment and beginning of the adjacent segment). In contrast, in the other system^[31-35] the impedance segments are arranged at an intersegmental distance of approximate 2 cm. Furthermore, the pressure transducers are located inside the impedance segment.

STUDY PROTOCOLS

The procedure is very similarly to standard manometry. The patients are asked to fast for at least 8 h before recording. In the sitting position, the recording assembly is passed nasally and positioned with all sensors in the stomach. The intragastric pressure is set as baseline pressure. With the subject lying in the supine position and after a 10-min accommodation period, a station pull-through is performed to accurately locate the LES

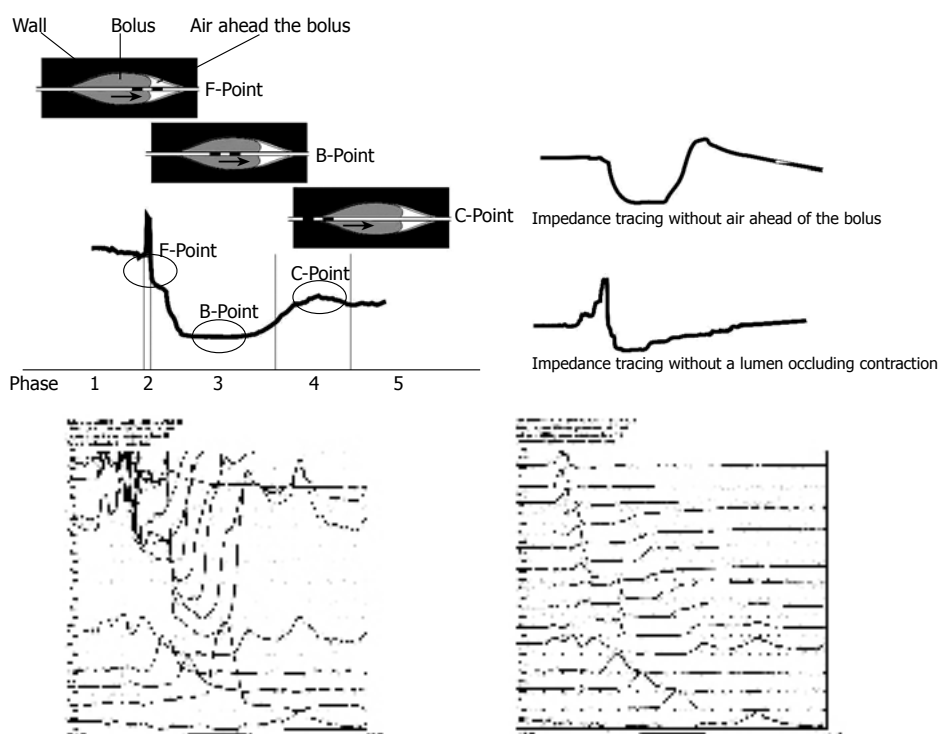


Figure 2 Upper panel: Characteristics of the impedance tracing during bolus passage in the esophagus of healthy persons. The black electrodes indicate a measuring segment. From baseline, the initial sudden impedance increase represents the arrival of ingested air ahead of the bolus. During the rapid impedance fall due to the arrival of the bolus, the bolus head (F-Point) can be determined as the return of impedance closely to baseline. The bolus passage is represented by a further decrease of impedance followed by a plateau phase. During the plateau phase (phase 3) the bolus is located within the measuring segment. The minimum impedance value during this phase is related to the maximum bolus volume located within the segment, which represents the bolus body (B-Point). Subsequently impedance increases due to wall contraction and the bolus is leaving the segment. The maximum value during impedance increase back to baseline is associated with the moment of lumen occlusion (C-Point). Dependent on bolus volume and bolus viscosity the exact position of the points of interest is variable as indicated by the oval circle. The impedance tracing may be variable as shown at the right side without air ahead of the bolus or without a rapid lumen occluding contraction. Lower panel: For visualisation of the minimum and maximum impedance an individual scaling (left side) can be used instead of a normal scaling (right side).

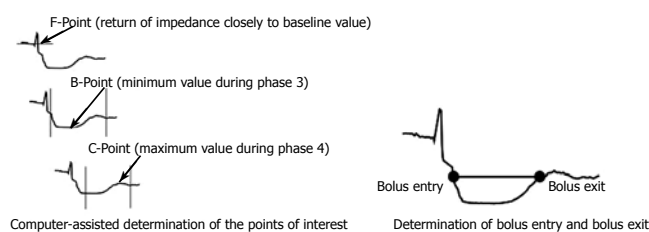


Figure 3 Upper panel: computer assisted determination of the points of interest according to presumed definitions as suggested by Nguyen *et al*^[24,27,29]. Lower panel: bolus entry and bolus exit can be considered to be 50% of the basal impedance as compared to nadir impedance as suggested by Tutuian *et al*^[32,33,34].

position. The most distal pressure sensor is placed at the level of the LES at the point at which the highest sphincter pressure is obtained during the pull-through procedure, the so-called lower esophageal high-pressure zone (LEHPZ).

To evaluate esophageal peristalsis and its associated bolus transport, liquid boluses (5 mL or 10 mL physiological saline) and semisolid boluses (5 mL standard viscous material provided by Sandhill Scientific Inc. or 10 mL of a commercially available plain yogurt, Morbo, Borken, Germany) are administered. The boluses are dispensed into the mouth with a syringe and the swallows are performed on command. All swallows are separated by at least 30 s during which no esophageal peristaltic occurs. An event marker is used to denote each swallow event. When a second swallow is incidentally initiated within 20 s of the primary event, both swallows are excluded from analysis. For each investigation, the baseline impedance is determined as the first predeglutitive impedance. After each yogurt bolus, at least 2 swallows of water are administered to clear small amounts of bolus material attached to the probe, until the impedance returns closely to baseline ($\leq 5\%$ deviation).

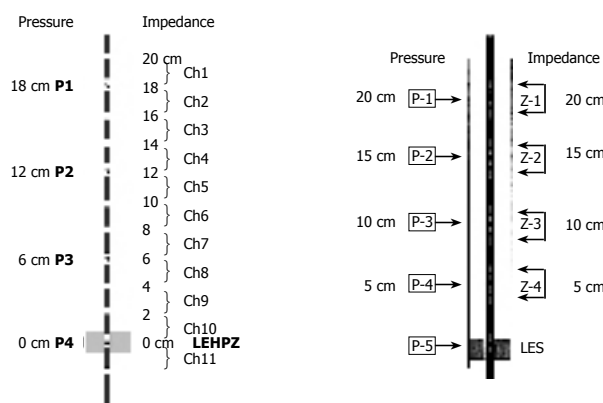


Figure 4 The 2 recently available systems of combined impedance manometry. During esophageal motility testing the most distally located pressure transducer was positioned at the lower esophageal high pressure zone. Left panel: 15 channel catheter for combined impedance-manometry procedure. The 4 semiconductor solid-state pressure transducers (P1-P4) serve also as impedance electrodes and are placed at 6 cm distance each. There are 11 impedance segments, each 2 cm long (Ch1-Ch11) with a cascade configuration. The solid-state pressure transducers (P1-P4) are located exactly between the impedance channels 1-2, 4-5, 7-8 and 10-11, respectively. Right panel: 9-channel esophageal function testing catheter with five pressure sensors and four impedance-measuring segment. The impedance measuring-segments consist of pairs of metal rings placed 2 cm apart, centered at 10, 15, 20 and 25 cm from the tip (Z1-Z4). Four of five the pressure sensors are located within the impedance segments (P1-P4). The 5th pressure sensors (P5) is located 5 cm from the tip.

PHYSIOLOGICAL OBSERVATIONS

With combined impedance manometry 3 different features of bolus transport can be obtained during swallowing (Figure 5): (1) monitoring of bolus transport patterns, (2) calculation of bolus transport parameters, and (3) monitoring of swallow associated events. These features can not be obtained by conventional techniques such as manometry or fluoroscopy.

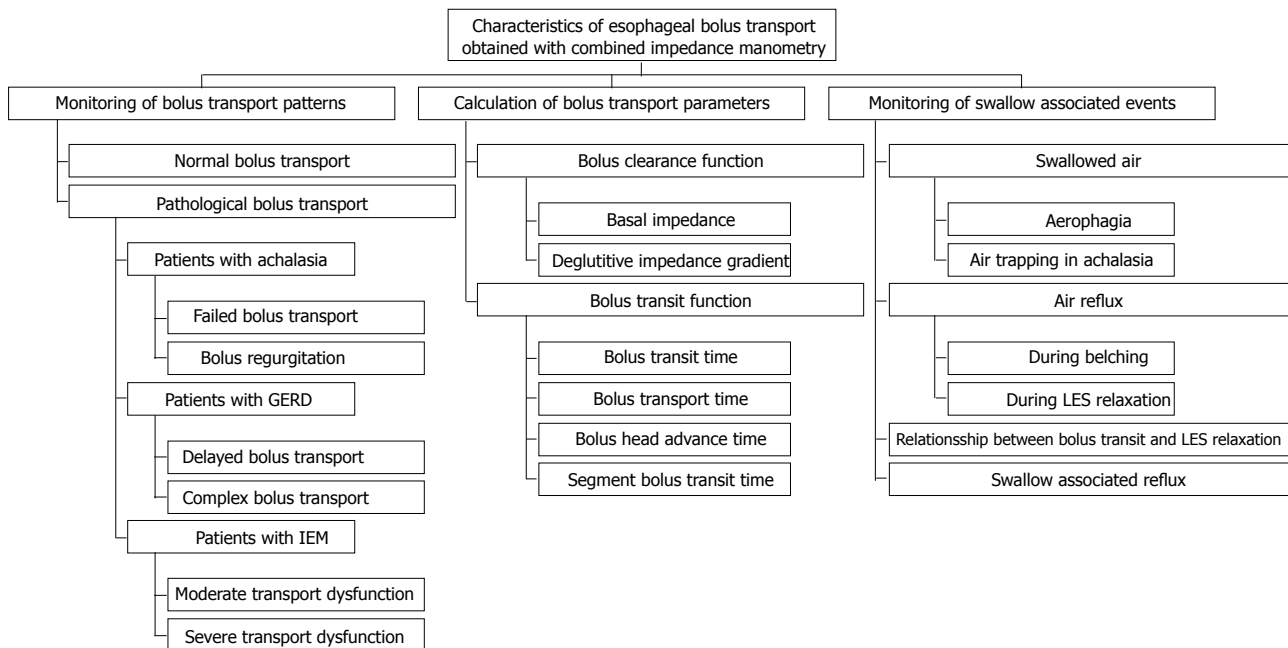


Figure 5 Characteristics of bolus transport as obtained by combined impedance manometry.

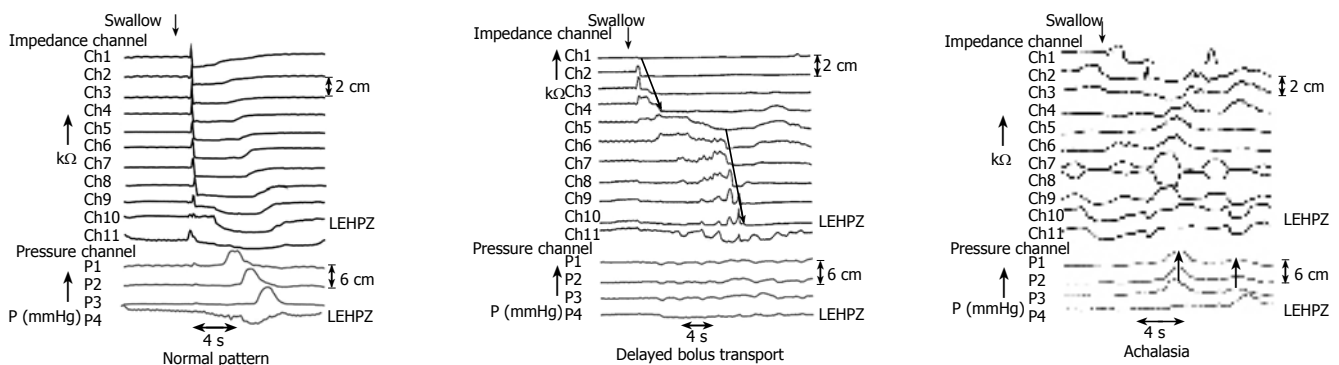


Figure 6 Bolus transport patterns as obtained by combined impedance manometry normal pattern (left panel), delayed bolus transport (middle panel) in some patients with GERD, failed transport (right panel) in patients with achalasia.

Monitoring of esophageal bolus transport patterns (Figure 6)

Recent results indicate that monitoring of bolus transport patterns may be helpful for the characterization of pathological esophageal bolus transport. (1) Healthy subjects show characteristic impedance patterns of continuous bolus transit through the esophagus into the stomach; (2) In patients with achalasia the manometry patterns are unique, while the impedance patterns are very variable^[28]. Different pathological patterns of bolus transport have been observed in patients with achalasia. They are (a) failed swallow-induced bolus transport through the esophagus in all cases, and (b) impedance evidence of luminal content regurgitation together with excessive air trapping. The results may explain some symptoms in achalasia, including chest pain and regurgitation; (3) In patients with GERD several pathological bolus transport patterns types have been described^[29], including failed bolus transport, delayed bolus transport, and complex bolus transport. The contribution of these phenomena and the clinical significance regarding to dysphagia symptoms remains to be determined.

Calculation of bolus transport parameters (Figure 7)

(1) Baseline impedance: It has been shown that there is a significant difference between gastric and esophageal baseline impedance in healthy subjects^[27] and a significant difference of esophageal baseline impedance between healthy subjects and achalasia patients^[28]. The results indicate that this parameter may be used to describe the esophageal resting state, including lumen width and filling state, particularly in minimal disease states. (2) Deglutitive impedance gradient: It has been shown that the postdeglutitive impedance is significantly lower in GERD patients as compared to healthy subjects, indicating that the deglutitive impedance gradient can be used to assess the propulsive esophageal clearance function^[29]. For correct calculation of this parameter, repetitive clearing swallows between test swallows are necessary^[29]. This parameter should be evaluated and standardized in further studies to determine its clinical significance for the definition of complete bolus transit. (3) Parameters of bolus transport: Several parameters can be calculated during impedance studies, i.e. propulsion velocity or transit time of bolus,

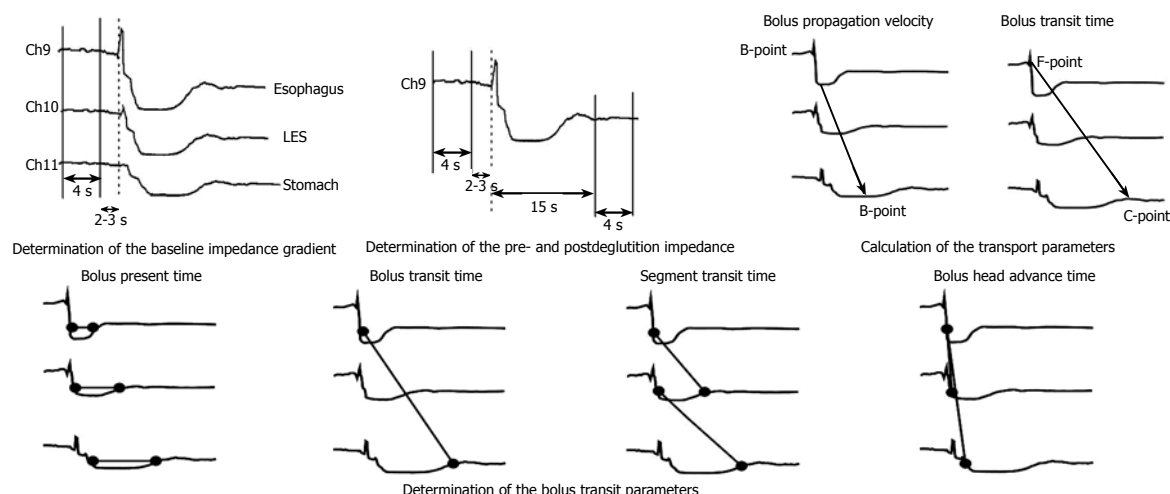


Figure 7 Bolus transport parameters, which can be obtained by combined impedance manometry according to presume definitions of the points of interest.

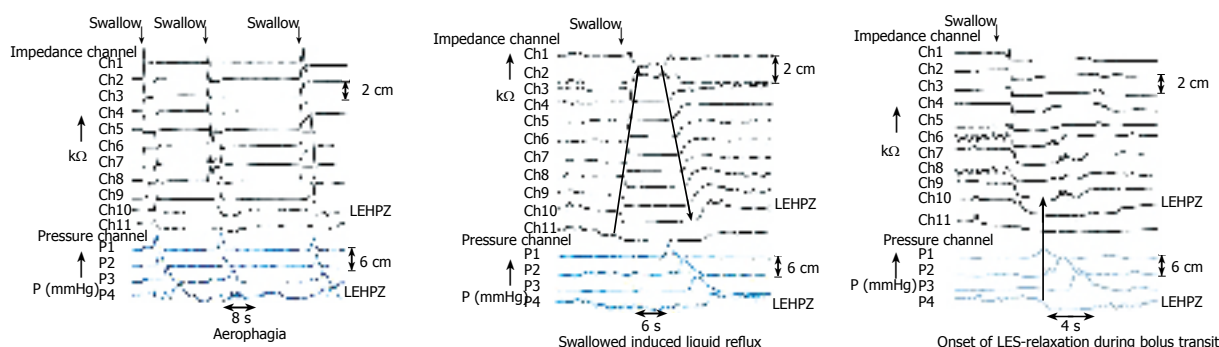


Figure 8 Swallow associated events as obtained by combined impedance manometry are aerophagia (left panel), swallow induced reflux (middle panel), relationship between bolus transit and LES-Relaxation (right panel).

bolus head and bolus tail^[24,27,29] or total bolus passage time, bolus head advance time, bolus presence time, and bolus transit time^[32-34]. The dynamics of the bolus transport along the esophagus has been described in detail^[24]. These parameters clearly discriminate liquid from semisolid boluses and show that bolus transport depends on both test substance and body position^[24,31,32]. (4) Quantitative data in healthy subjects indicate that impedance and manometry provide independent parameters of esophageal motor function^[27,33]. There is no clear correlation between contraction amplitude and bolus propagation velocity^[27]. As normal esophageal volume clearance may occur within a wide range of peristaltic amplitudes^[8], it is possible that after a critical pressure, excess amplitude does not appear to be needed to move the bolus. (5) Quantitative data in patients with mild GERD demonstrate delayed bolus transit in patients with non-erosive GERD and GERD grade I, indicating that combined impedance-manometry may increase the sensitivity for detection of minor motility abnormalities^[29]. (6) Quantitative data in patients with ineffective esophageal motility^[34] indicate that presumed “ineffective” low-amplitude esophageal peristaltic contractions of < 30 mmHg^[37] may be associated with a substantial number of complete transit: 23 patients (3.9%) had normal bolus transit for both liquid and viscous swallows, 12 patients (17.1%) had normal bolus transit

for liquid swallows and abnormal bolus transit for viscous swallows, and 10 patients (14.3%) had normal bolus transit for viscous swallows and abnormal bolus transit for liquid, whereas 25 patients (35.7%) had abnormal bolus transit for both liquid and viscous. The results indicate that combined impedance manometry better describes and classifies patients with ineffective esophageal motility according to severity: no functional abnormalities with normal transit, moderate functional abnormalities with abnormal transit either for liquid or viscous boluses, and severe functional abnormalities with abnormal bolus transit for both liquid and viscous boluses.

Monitoring of swallow-associated events (Figure 8)

(1) Movement of air during swallowing: In early studies it was shown that one advantage of the impedance technique is the monitoring of air movement during swallow and LES-relaxation^[24]. Recent studies have expanded the finding of esophageal air movement in patients with achalasia, so-called air trapping^[28], as well as during aerophagia^[36]. The clinical significance of this phenomenon as a cause of dysphagia remains to be determined. (2) Swallow-associated reflux: Different types of reflux have been observed in patients with GERD during swallowing^[29]: (a) liquid reflux initiated by a swallow and preceding the regular bolus transport, (b) liquid reflux

following a swallow-induced regular bolus transport, and (c) spontaneous liquid reflux initiating a clearing swallow. Since conventional 24-h pH-monitoring does not differentiate between fasting and deglutition associated reflux, the prevalence of these impedance phenomena and their relevance for mucosal damage have yet to be defined. (3) Relationships between bolus transit and LES relaxation: Our recent study^[30] revealed close relationships between bolus transit and LES relaxation. In 76% of the cases LES relaxation occurs during bolus transit, when the position of the bolus is very close to the LES. The results indicated that LES-relaxation may be partially initiated by bolus transit. The clinical significance of this finding for characterization of patients with LES-dysfunction other than achalasia - such as hypertensive LES or poorly relaxing LES - should be evaluated. Using high-resolution impedance monitoring, the opening patterns of the esophageal gastric junction during deglutition and transient lower sphincter relaxation have been studied recently^[37].

CLINICAL RELEVANCE

Clinical studies showed that combined impedance manometry is particularly suitable for comprehensive esophageal motility testing and monitoring of bolus transport patterns. With impedance different aspects of bolus transport can be obtained: (1) normal and pathological bolus transport patterns including bolus escape and retrograde bolus transport can be monitored, (2) several parameters of bolus transit can be calculated allowing differentiation between normal and abnormal bolus transport, (3) parameters related to bolus propulsive clearance and bolus transit completion can be determined, (4) swallow-associated events such as normal and pathological air movement as well as pathological reflux can be monitored, and (5) the relationship between bolus transit and LES relaxation can be investigated. Thus, using combined impedance manometry complete data about esophageal motor function and associated bolus transport can be obtained during a single investigation. In patients with achalasia the technique provides additional information about the functional status and may explain some symptoms in these patients. However, the gold standard for diagnosis of achalasia remains manometry due the clear definitions and the unique manometry patterns. In patients with GERD combined impedance manometry provides additional information about mechanisms related to disturbed bolus transit and bolus clearance. In patients with ineffective esophageal motility it helps clarifying the associated functional abnormalities. Thus, combined impedance manometry is on the way to be an important tool for obtaining detailed information about the physiology and pathophysiology of esophageal motility. The potential clinical implications of this technique include (1) the functional classification of esophageal motor disturbances in patients with non-obstructive dysphagia; (2) the perioperative management of laparoscopic fundoplication and other (endoscopic) antireflux procedures and (3) the evaluation of pharmacological approaches to esophageal motility and bolus transport.

FUTURE PROSPECTS

However, the studies also indicate that standardization of experimental set-up including equipment, study protocols, and particularly analysis algorithms with definition of the events of interest are important to make the data reproducible and the interpretation of the results more concise. There are important issues to be solved in order to make the technique more reliable and suitable for routine clinical use.

The equipment, particularly the spatial arrangement of impedance segments and pressure transducers should be clarified, because this is the most important determinant for the analysis of the relationship between manometric and impedance events during simultaneous monitoring. Since bipolar impedance measurements are performed between two electrodes, the impedance values obtained are the results of an integrative change of intraluminal electrical conductivity and cross-sectional area in the whole 2 cm segment. This should be kept in mind as an important aspect that differs from point manometry. At each corresponding impedance segment there is a 2 cm distance between the bolus entry point (first electrode) and the bolus exit point (second electrode). In case that the pressure transducer is located at the end of an impedance segment^[27,29,30], the impedance and manometric timing of bolus exit are approximately identical. If the pressure transducer is located inside the impedance segments^[33-35], the manometry bolus tail is not identical with the impedance bolus tail, as the bolus has not yet left the segment. The appropriate number of impedance segments is another issue. With both available systems several parameters of bolus transport can be obtained. The 15-channel system allows a detailed monitoring of bolus transport patterns and associated events^[24,27-30]. It remains to be determined, if these features are clinically significant. In contrast, the 9-channel system allows a significant reduction of data acquisition and reduces production costs, thus it is now available for routine clinical use^[31-34].

The analysis algorithm including the definition of bolus entry and bolus exit points should be refined. Since impedance is non-linearly dependent on different parameters around the electrodes such as lumen width and electrical conductivity of luminal contents, the interpretation of impedance tracings is based on impedance changes from variable levels and not from fixed calibrated points: (1) relative impedance changes as compared to baseline or extreme values (maximum and minimum) should be used in favour of absolute values as normally seen in manometry; (2) a small amount of a highly conductive substance may yield the same impedance change as a great amount of a low conductive substance, particularly if the conductivity of luminal contents is highly variable, i.e. gastric contents during reflux or duodenal contents during gastric emptying^[38,39], or when different test substances are employed^[27,29,33,34].

In the early studies^[24,27,29] the points of interests were determined visually with computer assistance according to a presumed definition of these points based on theoretical considerations and small validation studies^[15,16]. The analysis is time consuming and therefore not appropriate

for large routine studies. In later studies^[33,34], bolus entry is considered to occur at the 50% point between impedance baseline and impedance nadir during bolus passage, and bolus exit is determined as 50% point on the impedance recovery curve. This analysis algorithm is simplified and computerized calculation is possible. However, these conventions may have to be refined. Since the same level of impedance was used both for the bolus head (bolus entry) and the bolus tail (bolus exit), and both for liquid and viscous boluses, this definition implied that bolus geometry remains constant independent from bolus characteristics and that the forms of bolus head and tail are identical, or that the bolus is symmetric, respectively. However, fluoroscopic studies^[8] have demonstrated that both conditions are not relevant to real life. Considering the impedance tracing during a bolus passage, bolus entry is associated with a very rapid drop of impedance. In this setting, 5%-10% variation in impedance will not yield significant differences regarding the determination of the bolus head. Therefore, this convention appears to be suitable for determination of the bolus entry. In contrast during bolus exit, impedance increases slowly, and 5%-10% variation will result in significant differences regarding the determination of the bolus tail. Therefore, constant impedance levels may not be appropriate to describe bolus transport under various conditions, particularly for the definition of bolus exit as a parameter for completion of bolus transit^[33,34]. Further studies are needed to reach consent on this critical issue.

The study protocols should be standardized and include characteristics of the test substances (viscosity and volume) and information on body position, all of which have been identified as determinants of impedance in several studies^[24,27,31,32]. Considering the bolus viscosity, a commercially available test substance with constant viscosity and electrical conductivity should be used as standard^[33,34]. Considering the test volume, both 5 mL or 10 mL are used^[24,27,33,34]. Traditionally, the 5-mL test volume is used to induce an appropriate manometric esophageal response to a swallow. It has been shown that during deglutition different amounts of air are swallowed together with the bolus^[12], which may interfere with impedance recording. According to our personal experience, 10 mL seems to be an appropriate volume^[15,16], because the larger volume reduces air swallows that might interfere with impedance recording. Since body position affects the impedance of bolus transport^[24,32]-which seems to depend on the degree of inclination as a result of the addition of gravity to bolus propulsion-studies may be performed with subjects at supine or recumbent position to eliminate this gravity affect.

More clinical studies are required to prove if combined impedance manometry effectively helps to improve our management of patients with esophageal symptoms, as did manometry in patients with achalasia several decades ago.

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REVIEW

Does gastric atrophy exist in children?

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Abstract

Several clinical reports confirmed that gastric atrophy is a pathology not only limited to adult patients. In pediatrics, it is most often described in association with a *H pylori* infection but this bacteria does not seem to be the only etiological factor of this preneoplastic state in children. The frequency of gastric atrophy and intestinal metaplasia in children are unknown because they are not systematically sought during upper gastrointestinal endoscopy. The lack of specific histological classification of children's gastropathies makes their diagnosis difficult for pathologists. Based on our knowledge to date, we think that it is necessary to describe, in detail, the natural course of this lesion during childhood. A close and prolonged clinical and endoscopic follow-up is important for children with gastric atrophy.

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Key words: Gastric atrophy; Gastritis; *H pylori*; Intestinal metaplasia; Gastric cancer; Children

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INTRODUCTION

The discovery of *H pylori* in adult patients by Warren and Marshall^[1] was a major event in modern gastroenterology, rewarded with the Nobel Prize in 2005, and strongly stimulated paediatric studies focused on gastroduodenal pathology. The presence of this microorganism in children's stomachs was described several years after it was found in adults. In fact, up to that time, gastric biopsy had never been a priority for paediatricians because of the

low frequency of gastroduodenal pathologies in children and the non existence of gastric cancer at this age of life. On the contrary, gastric atrophy is systematically sought for in adult stomach biopsies because of cancer pathology. Its histological diagnosis is based on the updated Sydney System^[2]. During the last few years, several publications reported the presence of this preneoplastic state in children and the search for *H pylori* during endoscopy contributed to the improved knowledge of histological lesions of gastric mucosa in pediatrics. However, uncertainty persists regarding histological criteria of chronic gastritis in children, its long term course, its relationship with intestinal metaplasia in adults^[3,4] as well as the responsibility of other etiological agents other than *H pylori*.

GASTRIC ATROPHY IN PEDIATRICS

Several clinical reports confirmed that gastric atrophy is a pathology not only limited to adult patients^[5-9]. However, because of the non-systematic search during pediatric gastroscopy for this histological state considered as preneoplastic in adults^[3,4,10,11], its prevalence is not well evaluated in pediatrics, varying from 0 to 72% according to different studies (Table 1). Moreover, published clinical data refer mainly to *H pylori*-infected children and therefore, the prevalence of gastric atrophy due to other etiologies is unknown at this time. Finally, the reported prevalence of gastric atrophy and intestinal metaplasia (IM) in pediatric studies includes all histological grades, whereas in the majority of adults' studies only medium and severe grades are considered; this could be a possible source of overestimation.

A recent Turkish study reported prevalence of 72% of gastric atrophy in a small series of 18 *H pylori*-infected patients^[6]. This study was performed in a country with a very high *H pylori* prevalence ranging from 43.9% to 53% and a relatively high age of the recruited patients (median age of 12.2 years) which incites one to think that such a severe mucosal alteration could be related to a prolonged disease duration, in predisposed individuals^[6,16], as it has been suggested in adults^[17,18]. However, a selection bias cannot be excluded because another Turkish group, which examined gastric biopsies of 175 *H pylori* positive children, found only five cases of gastric atrophy and/or IM corresponding to a lower prevalence of 2.8%^[8]. Authors of studies carried out in other countries, who focused on *H pylori*-positive children, reported gastric atrophy in a very limited number of cases varying from 0 to 4%^[13,14,19,20].

Gastric atrophy of young children was only described in five cases^[5,7]; two were one year-old infants and two others were two year-old patients. Those observations do

Table 1 Studies concerning atrophic gastritis in children

Authors	Updated Sydney system	Total of included patients	Average age of included patients (yr)	Hp+ (n)	Prevalence of gastric atrophy (and/or IM) ¹ (%)	Average age of patients with GA (yr)	Number of patients < 10 yr with GA
-Whitney ^[5] 2000 USA	Yes	42	11	42	19% Hp+ (¹ 0%)	11	4
-Kolho ^[12] 2000 Finland	Not reported	71	9	71	0% (¹ 0%)	Not reported	0
-Cohen ^[13] 2000 Argentina	Yes	15	11	15	0% (¹ 0%)	Not reported	0
-Campbell ^[14] 2001 Gambia	Yes	37	2	21	0% (0%)	Not reported	0
-Ozturk ^[6] 2003 Turkey	Yes	27	12	18	72% Hp+ and 11% Hp- (¹ 78% all Hp+)	Not reported	Not reported
-Guarner ^[7] 2003 USA	Yes	64	Hp+=9 Hp-=9	19	63% Hp+ and 22% Hp- (¹ 21% all Hp+)	8 (Hp+)	6
-Usta ^[8] 2004 Turkey	Yes	175	12	175	2% Hp+ (¹ 2%)	12	1
-Levine ^[9] 2004 Israel	Not reported	95	14	55	1% 1Hp- (0 Hp+) (¹ 0%)	14	0
-Kato ^[15] 2006 Japan	Yes	196	11	131	- antral GA : 52% Hp+/11% Hp- (5% Hp+/5% Hp-) - fundic GA : 35% Hp+/8% Hp- (0% Hp+/4% Hp-)	Not reported	Not reported

¹IM: Intestinal metaplasia. Hp: *H pylori*

not call into question the hypothesis of Correa *et al*^[4] about the progressive, stepwise installation of precancerous lesions but they incite one to think that individual susceptibility to those premalignant lesions probably exists. In fact, it is probable that genetic and environmental factors do not only participate in the pathogenesis of gastric atrophy in adults^[21] but also in children. This was shown in the study by Campbell *et al*^[14], which was carried out within the West African population where the infection rate with *H pylori* is one of the highest in the world (70% to 90%), yet did not describe any cases of gastric atrophy. This phenomenon was called “the African enigma”. To our knowledge, there is no pediatric study, comparing by using the same methodology, the prevalence of gastric atrophy in a population according to its geographic/genetic origins or environmental factors.

Cases of intestinal metaplasia in stomach, which is also considered as a precancerous lesion in adults^[3,4,10,11], were more rarely reported in pediatric patients^[6,8,15] (Table 1). According to some authors, intestinal metaplasia is never associated to *H pylori* infection^[5,13,14,19], whereas others point out its frequency to be lower than 5% among infected patients^[8,15,20,22]. In contrast with this very low rate, Guarner *et al*^[7] reported a prevalence of 21% which brings one to think that an obvious relationship exists between *H pylori* gastritis and intestinal metaplasia.

ARE SPECIFIC HISTOLOGICAL CRITERIA FOR PAEDIATRIC ATROPHIC GASTRITIS AVAILABLE?

Up until now, no specific histological classification of children's gastropathies has been validated^[23]. By extension with adult gastropathies, in clinical practice, the Updated Sydney System is widely used^[2]. Its main

purpose is to allow for pathologists to establish a severity classification by visual comparison between the sampling and the published diagrams by Dixon *et al*^[2]. This essential publication defines gastric atrophy as glandular loss of mucosa which is replaced either by fibrous tissue or by intestinal metaplastic cells. However, the interposition of inflammatory cells between the stomach's glandular cells may inappropriately orientate the pathologist to the diagnosis of atrophy, where one is not present. Sampling interpretation is observer-dependent and this was emphasized by several studies^[13,24,25]. Without strict validated criteria, the severity assessment of gastric atrophy remains subjective and not easily reproducible^[26].

The authors of the same study recommend to perform five gastric biopsies in adults (2 antral, 2 corporeal and one from the angulus) for histological analysis but no consensus is available about the optimal number of biopsies needed in children. Clinical practices in this domain are very heterogeneous, which may be responsible for the underestimation of the prevalence of atrophic gastritis in children. To this end, Bedoya *et al*^[26] performed only two gastric biopsies in the diagnosis of 175 cases of *H pylori* gastritis, whereas Guarner *et al*^[7] as well as Derambure-Wizla *et al*^[27] carried out 3 or 4 biopsies for series of 64 and 436 children, respectively. In light of these studies and the Dixon's recommendations^[2], it is probable that the multiplication of gastric biopsies is an essential factor for a positive diagnosis and severity assessment of gastric atrophy in children.

Regarding intestinal metaplasia with a patchy distribution throughout the gastric mucosa^[27], the situation is similar and it may be easily missed if not enough gastric biopsies are taken. In contrast to gastric atrophy, metaplastic epithelium is easily detected by the pathologist, owing to the very characteristic goblet cells^[2].

However, endoscopy for younger children cannot be

as frequently performed as in adult patients. Less invasive procedures for detecting histological modifications of gastric mucosa could be valuable in the clinical practice. In adult patients, it has been shown that gastric atrophy was responsible for loss of production of hydrochloric acid, decreased serum levels of pepsinogen I and increased gastrin serum levels^[28]. Probably, hypergastrinemia could be a valuable marker for gastric atrophy in pediatrics, in order to select patients for further investigation by gastroscopy^[29]. It was also hypothesized that low levels of gastric acid secretion were a factor in the development of gastric atrophy in adults^[30] but no evidence for this hypothesis was published regarding pediatric patients. Due to the low prevalence of gastric atrophy during childhood, it would be difficult to validate these potential screening tests for children.

ARE THERE SPECIFIC FEATURES FOR CHILDREN'S GASTRIC ATROPHY?

Even though reported frequencies of gastric atrophy and intestinal metaplasia in children are extremely variable depending on authors (Table 1), they are definitely lower than those in adults^[31-35], thus some authors do not confirm their existence^[22,36,37]. It seems that in the series where the median age is higher, the prevalence of gastric atrophy is also increased. It is plausible that the duration of *H. pylori* infection in childhood may explain this phenomenon^[38].

No specific clinical symptoms are noted in adults and children. In most pediatric cases, its diagnosis is established in relationship with upper gastrointestinal symptoms^[39], but the prevalence of asymptomatic cases is unknown.

The main histological differences between atrophic gastritis of children and adults seem to be related to the characteristics of inflammatory response accompanying mucosal atrophy. In most series comparing children and adults, the degree of gastric mucosa colonisation by *H. pylori* seems to be significantly more important in children. However, the severity of the inflammatory response remains controversial depending on the authors' findings. Whitney *et al*^[5] reported a higher gastritis activity in adults compared to children, whereas Meining *et al*^[22] concluded that gastric inflammation is very severe in the pediatric age group. The absence of standards to measure inflammation could explain those differences. This highlights the importance of generalising the use of the Updated Sydney Classification for the assessment of gastric inflammation (not used by Meining). Finally, the study by Campbell *et al*^[14] carried out in Western Africa, using the Updated Sydney System, confirms some of the conclusions by Whitney: severity of gastric inflammation is more important in adults than in children, while differences in the degree of mucosal colonization by *H. pylori*, between children and adults, was not found.

IS *H. PYLORI* THE ONLY RESPONSIBLE AGENT FOR GASTRIC ATROPHY IN CHILDREN?

The role of *H. pylori* as a main etiological agent in atrophic gastritis is indisputable. However, several observations of

Table 2 Studies reporting observations of atrophic gastritis in children without *H. pylori* infection

Authors	Total of cases Hp+ (n)	Hp- (n)	atrophic gastritis Hp- (n)	Clinical symptoms (average age)
- Ozturk ^[13] 2003 Turkey	18	9	1 mild	Chronic abdominal pain (12 yr)
- Guarner ^[12] 2003 USA	19	45	10	Upper chronic digestive symptoms (9 yr)
- Levine ^[14] 2004 Israel	55	40	1	Gastro-esophageal reflux, epigastric pain, collagenous gastritis

Hp: *H. pylori*.

gastric atrophy in children without infection are reported^[6,7,9] and Table 2). It seems logical to suspect the existence of other etiological factors responsible for gastric atrophy. In adults, Kuipers *et al* reported that patients receiving omeprazole for long periods of time presented with a higher risk to develop gastric atrophy, yet in their series, all patients were *H. pylori*-positive^[40,41]. Those data were not confirmed in more recent studies^[42-44] and to date, proton pump inhibitors (PPI) are not considered as responsible for gastric atrophy in adults. To our knowledge, no cases of gastric atrophy attributable to PPI in pediatrics have been reported.

The hypothesis of an autoimmune disorder responsible for the appearance of antigastric antibodies was confirmed in adults. The frequency of those antibodies in patients with gastric atrophy may be as high as 50%^[45,46]. It has been reported that in some cases of severe gastric atrophy in adults, previously infected by *H. pylori*, any infection stigmata may disappear when anti-gastric-parietal cell antibodies appear^[47,48]. The authors of those observations suggested that anti-gastric-parietal cells antibodies could be a biological marker for the severity of gastric atrophy. In contrast, very few pediatric data are available. The only clinical situation where anti-gastric-parietal cell antibodies were discovered was type I diabetes with a frequency of 6% but no cases of atrophic gastritis have been reported^[49-51]. Celiac disease is another cause of chronic gastritis with positive findings of anti-gastric-parietal cell antibodies in 10% of cases in adults^[52], but similarly, no pediatric data are available. The study by Kolho *et al*^[12] included sixty children with celiac disease but no cases of anti-gastric-parietal cell antibodies have been pointed out. Nevertheless, several anecdotic cases of autoimmune thyroiditis and juvenile hypothyroidism, some of which with achlorhydria and gastric antibodies have been reported^[53].

LONG TERM COURSE OF GASTRIC ATROPHY IN CHILDREN

The long term course of gastric atrophy in children is unknown. To our knowledge, no longitudinal study of this course, through to adulthood, has been published.

In adults, Correa *et al* suggested that chronic gastritis, gastric atrophy, intestinal metaplasia, dysplasia and gastric cancer develop stepwise over decades, in predisposed individuals infected by *H pylori*^[2,3]. However, observations of atrophic gastritis have been reported in very young children (Table 1), including the presence of intestinal metaplasia in pediatric patients. Therefore, the chronological sequence of appearance of those lesions is not respected in all the cases. The association of intestinal metaplasia with *H pylori* infection is variable and it is impossible to prove that intestinal metaplasia is preceded by gastric atrophy in all cases. The risk of cancer in adults grows proportionately with the histological progression of metaplasia (evaluated from I: mild to III: according Dixon *et al*^[54]). Gastric cancer is exceptional in pediatric population and only one case has been reported, involving a 15 years old boy who was not infected by *H pylori*^[55].

It is actually well established that only 1% of *H pylori*-positive patients will develop gastric cancer^[56], whereas infection starts almost all the time during childhood^[57-60]. The rarity of intestinal metaplasia and gastric cancer in the pediatric age group could probably be explained by the long time period necessary for those complications to appear as well as the absence of other cumulative factors for gastric cancer, during childhood.

Probably insufficient ingestion of vitamin C could contribute to the development of *H pylori* infection in children^[61] and in adults^[62-64] but its role in the genesis of gastric precancerous lesions is not clearly established^[63,65]. Moreover, the question of the spontaneous reversibility of gastric atrophy after *H pylori* eradication is frequently discussed.

It seems difficult or even impossible, because of evident ethical barriers, to investigate whether the eradication of *H pylori* during childhood would decrease the risk of gastric cancer during adulthood^[66].

CONCLUSION

Gastric atrophy exists in children and it is sometimes found in very young children. It is necessary to describe, in detail, the natural course of this lesion during childhood. A close and prolonged clinical and endoscopic follow-up is necessary for children with gastric atrophy. The efficiency of preventive strategy^[66] or screening of *H pylori* infection in evaluating the risk of gastric atrophy and cancer in adult age should be evaluated. Finally, it is necessary to identify, by means of multicentric studies, the other circumstances (excluding *H pylori*) predisposing to gastric atrophy and its evolutionary potential over decades.

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GASTRIC CANCER

p21 and p27 immunoexpression in gastric well differentiated endocrine tumors (ECL-cell carcinoids)

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more studies are needed to assess the role of these prospective markers in gastrointestinal endocrine tumors.

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Abstract

AIM: To investigate the expression of cyclin-dependent kinase inhibitors p21 and p27 in gastric well differentiated endocrine tumors (GWDET) (ECL-cell carcinoids).

METHODS: The expressions of p21 and p27 were examined immunohistochemically in endoscopic biopsy specimens from 16 patients matching the diagnostic criteria of GWDET. Percentage of positive nuclear staining either weak or strong was noted. The association of immunoexpressions with age, gender, tumor localization, multifocality and accompanying chronic atrophic gastritis, neuroendocrine cell hyperplasia (NEH), neuroendocrine dysplasia (NED), intestinal metaplasia (IM), Ki-67 proliferation index and clinical outcome were also evaluated.

RESULTS: All cases expressed p27 with a mean expression score of 43.6%, while 31.3% of the cases showed any p21 expression. p21 and p27 immunoexpressions were significantly correlated with each other ($P < 0.01$), and the p21-expressing group had higher p27 expression scores (68% vs 22%). p21 and p27 expressions were lower in women, in non-atrophic mucosa and cases whose tumors were located somewhere other than fundus without submucosal extension. On contrary, p21 and p27 expressions were higher in males and the patients with submucosal extension and atrophic gastritis. Cases presenting lower p27 scores had solitary tumors showing neither NEH-NED nor IM. Despite, cases with lower p21 expression presented multifocal tumors accompanied by NEH-NED. However, no correlation of p21 and p27 expressions was found with age and Ki-67 expression.

CONCLUSION: p27 is widely expressed in GWDETs, while p21 expression is sparse and observed in two thirds of the cases. Loss of p21 and p27 expressions may be correlated with different carcinoid tumor subtypes; however,

INTRODUCTION

Carcinoid tumors arise from proliferating enterochromaffin-like cells (ECL) of fundus. Elevated plasma gastrin levels are responsible of the neoplastic changes in these cells, but neoplastic transformation can also be observed in absence of hypergastrinemia as well. The overall incidence rates for carcinoid tumors have increased significantly over the past 25 years^[1]. Recent studies also suggest an apparent increase in incidence of gastric carcinoids which accounted about 2% of the total gastrointestinal (GI) carcinoids in the past but increased to a frequency of 7.2% to 30% in recent years^[2-5]. Increased awareness of the existence of these lesions as well as the improvements in upper GI endoscopy and more frequent application of immunohistochemical methods to biopsies are probably related to this trend. The causes of this inclination need further investigations, but the conclusion is clear that we have to cope with more endoscopic biopsies with neuroendocrine cell hyperplasia and tumors. The role of the pathologist is not only to diagnose the lesion but also provide the histopathologic data that will help to determine the management strategies.

Well differentiated endocrine tumor (WDET) is proposed as synonymous with the time-honoured term "carcinoid" according to the last WHO classification of the endocrine tumors of the GI tract (Table 1) and defined as "an epithelial tumor of usually monomorphous endocrine cells showing mild or no atypia and growing in the solid nests and trabeculae or pseudoglandulae, restricted to the mucosa or submucosa"^[6]. Further WDET's of stomach are ECL-cell carcinoid, EC-cell, serotonin-

producing carcinoid, G-cell gastrin-producing tumors and others^[6]. ECL-cell carcinoids clinically has three distinctive types: Type 1 which is associated with chronic atrophic gastritis with or without hypergastrinemia; Type 2 with hypertrophic gastropathy with high gastrin levels; and Type 3, sporadic form, associated with neither atrophy nor hypergastrinemia^[7]. Gastric well differentiated endocrine tumors (GWDET) are benign, non-angioinvasive tumors, measuring ≤ 1 cm in size and showing ≤ 2 mitoses per 10 high power field (HPF). The preferred therapy is endoscopic removal, followed by regular endoscopic surveillance and antrectomy in hypergastrinemic cases^[8].

In daily practice, type 1 ECL-cell carcinoids are more frequently observed than other subtypes. Despite their benign nature, recurrence is one of the leading problems in management of these tumors^[9]. Besides, lymph node metastases, although rare, are reported even in minute lesions^[10]. Furthermore, the frequent recurrences lead to more aggressive surgical approaches. So the prediction of tumor behavior from endoscopic biopsies is very critical, but it is quite difficult, if not impossible. In most of the cases, biopsy specimens are very small and submucosa is rarely visualized. Even when the submucosa is present, it is generally not deep enough to rule out submucosal invasion. Cytomorphologic features are helpless since the tumors do not show nuclear pleomorphism, anaplasia and frequent mitosis. Ki-67 proliferation index (PI) and p53 has clinical implications to some extent but new markers are needed to understand the mechanisms of tumor development and differences in clinical behavior even in benign lesions.

p21^{waf1} (p21) and p27^{kip1} (p27) are nuclear proteins reported to have a role in development and progression of several organ tumors. They are members of the KIP subgroup of cyclin-dependent kinase inhibitory (CDKI) family, which prevents cell entry to S phase in response to DNA damage, thus act as tumor suppressors. Loss of p27 has a role in carcinogenesis of several organ tumors, including breast, prostate and colon, and is related with poor prognosis^[11]. p27 expression has been reported to be valuable in differentiating parathyroid adenomas and carcinomas^[12,13]. Altered patterns of p21 expression seem to have an association with clinical outcome in certain tumors but there are conflicting results whether low or high levels of p21 serve as a prognostic marker^[13,14]. Although there are previous studies on the role of CDKIs in several types of endocrine carcinomas, the expression of these proteins in GI endocrine tumors, particularly GWDETs, is less well investigated^[15,16].

In this study, we first investigated expressions of p21 and p27 in GWDETs, and then evaluated the association of p21 and p27 expressions with age, gender, tumor localization, multifocality and accompanying chronic atrophic gastritis, as well as neuroendocrine cell hyperplasia (NEH), neuroendocrine dysplasia (NED), intestinal metaplasia (IM), Ki-67 PI and clinical outcome.

MATERIALS AND METHODS

Subjects

Formalin-fixed, paraffin-embedded endoscopic biopsy

Table 1 Gastric endocrine tumor classification, WHO^[6]

Classification	Diameter	Ki-67 PI ¹	Mitosis	Extension
Well differentiated endocrine tumor (carcinoid tumor)	≤ 1 cm	$\leq 2\%$	$\leq 2/10$ HPF ²	Mucosa-submucosa
Well differentiated endocrine carcinoma (malignant carcinoid)	> 1 cm	$> 2\%$	$> 2/10$ HPF ²	Muscularis propria and beyond
Poorly differentiated endocrine carcinoma (small cell carcinoma)	Any diameter	$> 15\%$	$> 10/10$ HPF ²	Vascular and neural invasion

¹Proliferation index; ²High power field.

specimens from 16 patients, whose archival materials were available for immunohistochemistry and matching the diagnostic criteria of GWDET^[6], were included in the study. The biopsy samples were fixed in 100 mL/L neutral buffered formalin. Following routine tissue process, tissues were cut into 4- μ m thickness and stained with hematoxylin & eosin and chromogranin A. Age, gender, tumor localization, multifocality (defined as tumors seen in more than one samples taken from different parts of stomach or same part of the stomach but described as separate nodules in endoscopy report) and accompanying chronic atrophic gastritis, NEH, NED and IM were recorded. Size of the tumors (although smaller than 1 cm) could not be measured properly because of the fragmented nature of the biopsies.

Immunohistochemistry

Mouse monoclonal antibodies (mAb) anti-p21 (Neomarkers, MS 230-P, 200 mg/L, 1:40 dilution), anti-p27 (Neomarkers, MS 256-P, 200 mg/L, 1:50 dilution) and Ki-67 (Dako, M 7240, 1:150 dilution) were used for immunohistochemistry by streptavidin-biotin peroxidase method. Briefly, sections were incubated at 50°C overnight, deparaffinized in xylol and rehydrated through graded alcohol rinses. To enhance antigen retrieval, slides prepared for p21 staining were heated in 0.1 mmol/L EDTA buffer (pH 8) in a microwave oven (650 W for 5 min plus 450 W for 20 min) and those for p27 were immersed in citrate buffer (10 mmol/L, pH 6) and heated in a vacuum-pan for 2.5 min and cooled for 20 min. Endogenous peroxidase activity was blocked with 30 mL/L H₂O₂ for 5 min. The incubation time with monoclonal antibodies p27 and p21 was optimized as 30 min. Immunostaining of p27 and p21 was performed with the streptavidin-peroxidase method (Labvision, TM-125-HL), and alkaline phosphate-based visualization system. 3, 3'-diaminobenzidine (Dako, TA-125 HD) was used as a chromogen. Carcinomatous breast and colon tissue served as a positive control, whereas primary mAb was omitted for negative control sections. Stained lymphocytes were used as an internal control. Scoring was performed separately by two pathologists (BD, BS) who were blinded to the patient characteristics as well as each others scores. In each case, a total of 200 cells were counted in two randomly selected representative HPF areas. Percentage of positive nuclear staining either weak or strong was noted. The mean of the scores counted by each observer was accepted as expression score.

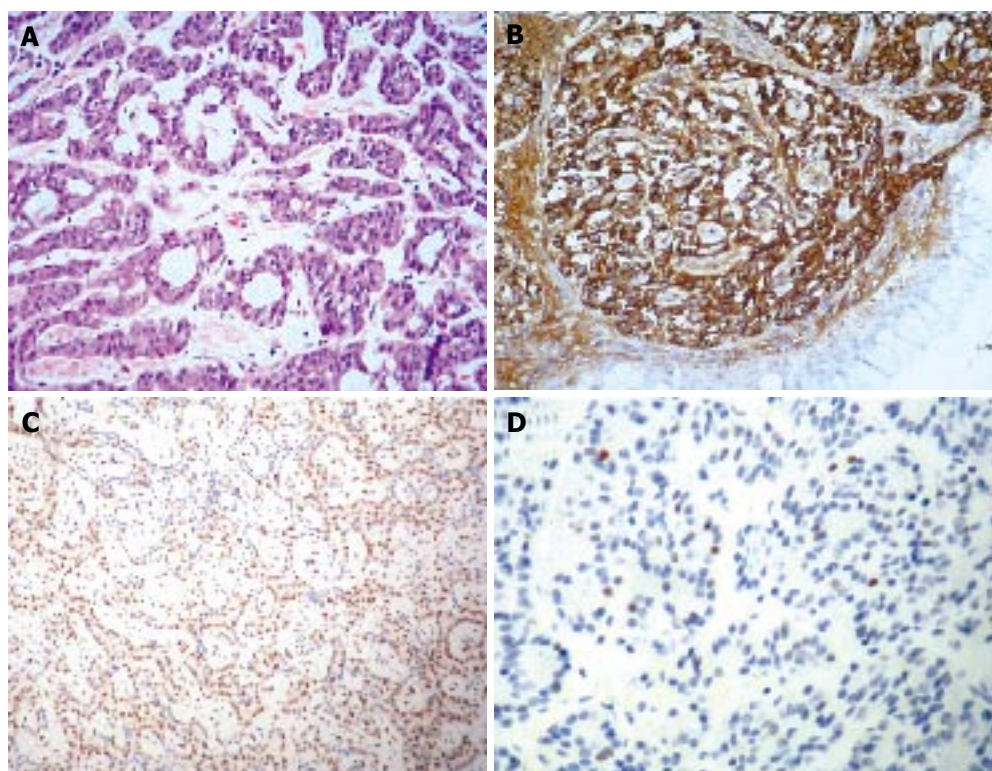


Figure 1 Histochemical staining. **A:** Tumor cells arranged in ribbons, tubular structures or small nests (HE x 20); **B:** Chromogranin immunoreactivity (x 10); **C:** p27 immunoreactivity (x 20); **D:** p21 immunoreactivity (x 20).

Table 2 Accompanying histopathologic features

Pathology	Patients (n)	Rate (%)
Atrophy	8	50.0
Neuroendocrine hyperplasia	7	43.8
Intestinal metaplasia	9	56.3
Neuroendocrine dysplasia	3	18.8
Multifocality	5	31.4
Submucosal extension	8	50.0

Statistical analysis

Statistical analysis was carried on a PC-based analysis program, SPSS 10.0. Pearson correlation analysis was carried out for univariate associations between p21 and p27 scores and age. Mann-Whitney *U* test was performed to investigate the relationship of p21 and p27 scores with gender, localization (fundus), multifocality, NEH, neuroendocrine dysplasia and presence of atrophy and IM as well as clinical outcome. Submucosal invasion, although enclosed in the definition of GWDET, was also noted when present and evaluated for its statistical significance by using Mann-Whitney *U* test. Chi-square test and Fisher's exact test were applied for dichotomized data. Student's *t*-test was used for associations with age. $P < 0.05$ was considered statistically significant.

RESULTS

Patient and tumor characteristics

Mean age of the patients was 52.4 ± 7.0 (range: 41-66) years, and 56.3% of them were female. All cases were classified as type 1 ECL-cell tumors clinically. Nine (56.3%) of the tumors were located in corpus and 2 (12.6%) of them were in fundus. Most of them showed more than

Table 3 p21, p27, Ki-67 staining ratios (%)

Gene	Mean \pm SD	Range
p21	1.09 ± 1.44	0-4
p27	43.58 ± 31.27	10-95
Ki-67	0.7 ± 0.94	0-3

one histologic pattern (Figure 1A). Chromogranin A was identified in all tumors (Figure 1B). None of the cases showed mitosis in 10 HPF except one case (1/10 HPF). Multifocality was observed in 5 (31.4%) cases. Accompanying histopathologic features are shown in Table 2. Eleven (68.8%) of the cases were cured by endoscopic excision, followed by regular endoscopic surveillance and endoscopic ultrasound when necessary and did not show recurrences during the follow-up. Among the rest of 5 (31.4%) cases, 3 cases needed re-excision and 2 were conveyed to surgery because of multiple recurrences. No additional histopathologic features were found in the operation materials of these 2 cases. None of the cases showed distant organ or lymph node metastasis at the time of diagnosis nor developed any during a mean follow-up period of 34.12 ± 25.63 (range: 9-69) mo.

p21 and p27 expressions

Descriptive statistics of p21, p27 and Ki-67 nuclear immunoreactivity are shown in Table 3, and Figure 1C, 1D. We observed a significant correlation between p21 and p27 expressions ($P < 0.01$, Pearson correlation analysis). All cases expressed p27, while 5 (31.3%) of the cases showed no expression for p21 in tumor areas. The p21-expressing group had higher p27 scores of 68% *vs* 22%, although statistically insignificant ($P = 0.07$) (Table 4). High p27-

expressing tumors (over the cut-off level of mean p27 expression of 43.6%) had higher p21 scores as well ($P = 0.022$, Mann Whitney *U*-test).

p21 and p27 expressions were higher in 3 tumors located in fundus either as a single focus ($n = 2$) or part of a multifocal spread ($n = 1$) ($P < 0.05$, Mann Whitney *U*-test) (Table 4). In addition, p21 and p27 expressions were higher in males and the patients with submucosal extension and atrophic gastritis. The patients with multifocal tumors, NEH and NED showed higher p27 and lower p21 immunoexpression. But no correlation of p21 and p27 expressions was found with the age and Ki-67 PI. Also, the p21-expressing tumors did not show any particular difference in respect to presence of atrophy, NEH, NED, IM, multifocality, submucosal extension, Ki-67 PI and age. Cases, who needed surgery or re-excision because of recurrence had lower p27 expression scores ($26.7\% \pm 4.2\%$ *vs* $50\% \pm 34.6\%$ in recurrent cases) and p21 immunoexpression ($0\% \pm 1.5\% \pm 0.53\%$ in recurrent cases), however, data did not research statistically significant. These cases did not differ in respect to presence of NEH, NED, atrophy, submucosal extension, IM, multifocality, gender and age.

DISCUSSION

The overall incidence rates of gastric carcinoids and related endoscopic biopsies have increased significantly over the last few years. Although a better prognosis can be predicted for most cases, the clinical outcome and management of these tumors vary widely^[2,4,17]. Determination of the most appropriate treatment modality and follow-up strategy depends on the associated or underlying disease process as well as the histologic findings in endoscopic biopsies.

Among the histologic parameters, Ki-67 PI is of diagnostic and prognostic value but it can be quite low in either benign or malignant neoplasms^[6,15]. In this study, the expression of two prospective prognostic markers, CDK1's p21 and p27, and their relationship with clinicopathologic features were investigated.

Cell cycle regulation is influenced by nuclear proteins that enhance cell division, cyclin-dependent kinases (CDK) or disrupt cell division, CDKIs. The induction of CDKI inhibits the activities of CDK2 and completely arrests cells at G1 phase of the cell cycle, thus acts as tumor suppressors^[18]. Loss of CDKIs has a role in carcinogenesis of several organ tumors as well as endocrine tumors. Canavese *et al*^[15] performed study on 109 endocrine tumors of the pancreas and GI tract, and found that p27 was highly expressed especially in differentiated tumors, while expression of p21 was very sparse in midgut (ileum-right colon) carcinoids but significantly higher in insulinomas^[16]. Zirbes *et al*^[19] stated that primary pulmonary carcinoid tumors failed to show a staining for p21 and p27.

We found that all cases expressed p27 with a mean expression score of 43.6%, while 31.3% of the cases showed p21 expression. p21 and p27 expressions were significantly correlated with each other and the p21-expressing group had a higher p27 expression score as 68% *versus* 22%. p27 has a 42% amino acid homology with p21, at the region that mediates inhibition of CDK^[12],

Table 4 p21 and p27 expressions and clinicopathologic features (%)

Clinicopathologic feature		p21	P ³	p27	P ³
Gender	Male	1.5	0.60	58.0	0.34
	Female	0.85		36.4	
Localization	Fundus	3.0	< 0.05	90.5	< 0.05
	Other	0.7		34.2	
Multifocality	Multifocal	0.7	0.57	45.7	0.92
	Single focus	1.25		42.9	
NEH ¹	Present	1.0	0.842	50.8	0.64
	Absent	1.16		37.5	
NED ¹	Present	0.7	0.57	45.7	0.80
	Absent	1.25		42.8	
Atrophy	Atrophic	1.3	0.47	46.4	0.50
	Non-atrophic	0.8		32.5	
Intestinal metaplasia	IM ² (+)	χ^4	χ^4	49.6	0.09
	IM ² (-)	χ^4		16.5	
Submucosal extension	Present	1.0	0.893	49.3	0.67
	Absent	0.8		30.5	
p27 immunoexpression	p ²¹ expressing	χ^4	χ^4	68.0	< 0.05
	p ²¹ (-)	χ^4		21.8	
Follow-up	Recurrent	0.0	0.094	26.7	0.51
	Non-recurrent	1.5		49.9	

¹Neuroendocrine cell hyperplasia-dysplasia; ²Intestinal metaplasia; ³Mann-Whitney *U* test; ⁴Result is omitted since p21 value was constant in IM (-) cases.

but recently it was suggested that p21, supposed to be functionally similar to p27, plays a lesser role in tumor suppression^[20]. But some studies suggest a co-regulation between p27 and p21 in some tumors like colorectal carcinoma and renal cell carcinoma^[21,22], but it is not yet clear how these two CDKIs relate to each other in carcinogenesis sequences.

In the present study, cases presenting clinical progression had lower p21 and p27 immunoexpression. But no particular difference was found in respect to presence of atrophy, NEH, NED, IM, multifocality, submucosal extension, and age. Ki-67 PI was very low in most of the cases and no correlation was found between p21, p27 expressions and the other clinicopathologic features. When we examined the general features of all cases, we found that both p21 and p27 expressions were lower in women, in non-atrophic mucosa and cases whose tumors were located somewhere other than fundus without submucosal extension. Cases presenting lower p27 scores were generally solitary tumors showing neither NEH-NED nor atrophy and intestinal metaplasia. Despite, in cases with lower p21 expression, tumors were multifocal and accompanied by neuroendocrine cell hyperplasia and dysplasia. These observations may suggest a probable association of p27 loss with sporadic form of the disease (Type III) and p21 loss with hypergastrinemic cases (Type I or II) although do not cover the whole picture.

Loss of p27 expression is reported to be correlated with aggressive behaviour of various organ tumors^[12,14]. But although Lloyd *et al*^[23] reported a marked decrease of 27 expression in benign and malignant endocrine tumors when compared with normal tissue and Erickson *et al*^[12] suggested the use of p27 in the differential diagnosis between parathyroid hyperplasia and adenoma, Canavese *et al*^[15] objected the use of high p27 expression as a marker of benign behaviour since it can be observed in differentiated

malignant tumors. Observation on p21 is more limited and needs further studies. To our best of knowledge, there is no previous study dedicated only to p21 and p27 immunexpressions in gastric well differentiated endocrine tumors. Although we appreciate that our study has limitations, the observations presented above can help to enlighten the pathogenesis of gastric carcinoid tumors. Larger series with long-term results are needed to assess the role of p21 and p27 in gastrointestinal endocrine tumors.

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Expression of insulin-like growth factor binding protein-2 in gastric carcinoma and its relationship with cell proliferation

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Abstract

AIM: To investigate the expression of insulin-like growth factor binding protein-2 (IGFBP-2) in gastric carcinoma and its clinical significance and to explore its relationship with cell proliferation.

METHODS: Expressions of IGFBP-2 and Ki-67 in 118 cases of gastric carcinoma and 40 cases of normal gastric mucosa were detected by EnVision immunohistochemical technique.

RESULTS: Expression of IGFBP-2 in gastric carcinoma was higher than that in normal gastric mucosa ($P < 0.01$). There was no difference between high- and low-grade gastric carcinoma ($P > 0.05$). Expression of IGFBP-2 in advanced gastric carcinoma was higher than that in early gastric carcinoma ($P < 0.05$). Expression of IGFBP-2 in gastric carcinoma with lymph node metastasis was higher than that without lymph node metastasis ($P < 0.01$). IGFBP-2 expression was a positively related to the clinical stage of gastric carcinoma ($P < 0.01$). There was a positive correlation between IGFBP-2 and Ki-67 ($P < 0.05$).

CONCLUSION: IGFBP-2 may be involved in carcinogenesis and progression of gastric carcinoma by promoting cell proliferation.

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Key words: Gastric carcinoma; Insulin-like growth factor binding protein; Cell proliferation; Immunohistochemistry

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INTRODUCTION

The insulin-like growth factor (IGF) system plays a crucial role in normal cell proliferation and malignant transformation^[1,2]. It comprises IGF- I and IGF- II, the type I and II receptors^[3], and a family of IGF binding proteins (IGFBPs) that specifically bind to IGFs^[4]. The IGFBPs are a family of binding proteins involved in the regulation of tissue development through their interactions with IGFs. By sequestering free IGFs and reducing their bioavailability for interaction with IGFs, IGFBPs are able to modulate cellular growth and differentiation at a local level^[5]. IGFBP-2 is the second most abundant IGFBP in the circulation and can be found in a variety of mammalian fluids and tissues. IGFBP-2 was initially thought to mainly bind to IGFs (IGF- I and IGF- II) through its IGF-binding motif, and thereby inhibits IGF-mediated mitogenic activities^[6]. However, recent studies have shown that IGFBP-2 also increases the tumorigenic potential and mitogenesis in some cancer cells^[7-9], suggesting that IGFBP-2 possesses multifaceted functions.

So far, only one study focusing on the expression of IGFBPs in gastric cancer cells^[10] is available. In this study, we used immunohistochemical method to clarify the expression and localization of IGFBP2 in malignant and normal gastric tissues. The aim was to determine whether IGFBP-2 plays a role during gastric carcinogenesis and to clarify the correlation of IGFBP2 expression with cell proliferation.

MATERIALS AND METHODS

Tissue samples and histopathological study

Paraffin blocks from gastrectomy specimens were obtained from the archives of Department of Pathology, Affiliated Yijishan Hospital of Wannan Medical College, between May 2004 and June 2005. Selected cases were classified as cardia carcinoma, fundus/body and antrum carcinomas according to their location, following the criteria described in a previous study^[11]. Mean age and gender were also recorded. Carcinomas were divided into two types, namely early and advanced carcinoma. The depth of wall penetration and nodal status were also recorded. The 1997 version of the International Union Against Cancer (UICC) pTNM system was employed to stage the carcinomas^[12].

Immunohistochemistry

Monoclonal antibody against IGFBP2 diluted 1:150 (c-18; Santa Cruz Biotechnology, Inc., Santa Cruz, UK) was used in immunohistochemistry studies. This antibody is specific and does not crossreact with other isoforms of IGFBP. For each procedure, the samples were chosen randomly from each group. The 4- μ m thick sections of phosphate buffered saline (PBS)/formalin-fixed and paraffin wax-embedded specimens were routinely dewaxed in xylene, rehydrated in a graded series of ethanol, and washed in distilled water. Antigen retrieval was achieved by placing the specimens in 0.01 mol/L citrate buffer at pH 6.0 and exposing them to repeated microwave heating for 10 min at 450 W. The buffer was replenished after each interval because of evaporation. The specimens were cooled at room temperature for 15 min and washed in sterile water for 5 min and then in PBS at pH 7.6 for 5 min. Endogenous peroxidase or phosphatase activities were quenched in 0.3% H₂O₂ for 30 min, followed by blocking non-specific antibody binding in 10% goat serum for 30 min at room temperature. Tissue sections were incubated overnight at 4°C in a humidifier with primary goat anti-IGFBP2. Mayer's hematoxylin nuclear stain was used as a counter stain. Negative controls were performed using conjugate alone.

Assessment of immunohistochemical staining

Immunohistochemical staining results were assessed semi-quantitatively using the Busund score system^[13]. Expression of IGFBP2 was then graded semi-quantitatively and classified into one of the four grades. The staining was scored as: 0: no staining; 1: weak staining; 2: strong staining of 25% tumor cells or moderate staining of < 80%; 3: strong staining of 25%-50% or moderate staining of > 80%; 4: strong staining of > 50% tumor cells. In each case, 10 high power fields of representative areas were counted. The maximal staining intensity was typically higher in those cases where more cells were positive. Most positive cells showed cytoplasmic staining. Slides were examined and scored independently by two of the authors, blinded to other pathological information. The Ki-67 labeling index (KI) was calculated as the percentage of positive tumor nuclei divided by the total number of tumor cells examined. At least 1000 tumor cells per specimen were examined in 5 randomly selected fields by light microscopy ($\times 400$).

Statistical analysis

Data are presented as mean \pm SD. Individual groups were compared using the parametric Student's *t* test. Association between IGFBP2 expression and different clinical parameters in malignant tissue was tested by nonparametric *t* test. The relation between IGFBP2 expression and Ki-67 was tested by Spearman's correlation coefficient. For all these statistical analyses, *P* < 0.05 was considered statistically significant.

RESULTS

Clinicopathological features

The clinical and pathological characteristics of 118 patients are summarized in Table 1.

Table 1 Clinical and pathological characteristics of patients with early gastric carcinoma (*n* = 118)

Characteristic	<i>n</i>
All patients	118
Gender	
Male/Female	71/47
Age (yr)	
Median (Range)	53.2 (24-76)
Tumor site	
Cardia	21
Fundus/body	43
Antrum	54
Histological type	
Differentiated	56
Undifferentiated	62
Lymph node metastasis	
Positive	67
Negative	51
pTNM stage	
I - II	52
III-IV	66
Depth wall penetration	
T1-T2	48
T3-T4	70

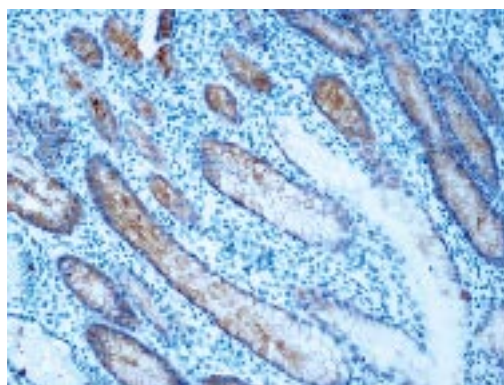


Figure 1 IGFBP2 expression showing a weak pancytoplasmic staining in normal gastric mucosa ($\times 200$).

Immunohistochemical expression of IGFBP2 in normal gastric mucosa and carcinoma

Among the 118 tumor samples, 40 specimens of normal mucosa (≥ 10 cm distant from cancer) showed a weak pancytoplasmic expression of IGFBP2 (Figure 1). The immunohistochemical staining score of IGFBP2 was judged as 0, 1 or 2. There was an over-expression of IGFBP2 in carcinoma. Pancytoplasmic staining or more diffuse granular staining was observed in cytoplasm of neoplastic cells (Figures 2 and 3). IGFBP2 expression score was 2.61 ± 0.94 in gastric carcinoma and 1.06 ± 0.64 in normal human gastric mucosa respectively. There was a significant difference between carcinoma and normal mucosa (*t* = 5.223, *P* < 0.01) as shown in Table 2.

Correlation between IGFBP2 expression and clinicopathological parameters of gastric carcinoma patients

A significant difference was observed in IGFBP2 expression and tumor depth of wall penetration (*P* = 0.019).

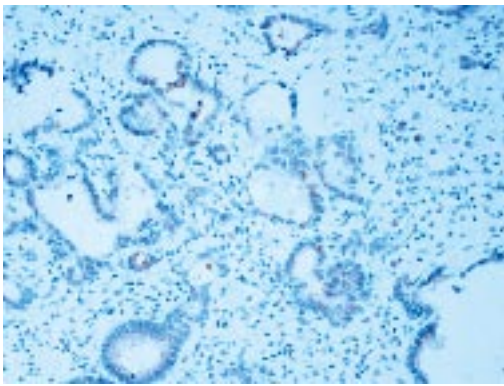


Figure 2 IGFBP2 expression showing a weak cytoplasmic staining in the tumor cells ($\times 200$).

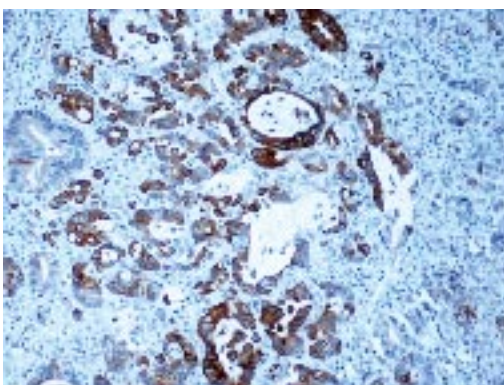


Figure 3 IGFBP2 expression showing a more diffuse granular cytoplasmic staining in the tumor cells ($\times 200$).

Table 2 Quantitative score of IGFBP2 expression in normal gastric mucosa and gastric carcinoma

Group	Quantitative score of IGFBP2 expression (mean \pm SD)	<i>P</i>
Normal mucosa	1.06 \pm 0.64	< 0.01
Carcinoma	2.61 \pm 0.94	

Although immunohistochemical staining score of IGFBP2 expression in undifferentiated carcinoma (2.72 ± 0.88) was higher than that in differentiated carcinoma (2.31 ± 0.82), the difference was not significant ($P = 0.336$).

A significant difference was also observed in IGFBP2 expression and nodal metastasis ($P = 0.0048$). The immunohistochemical staining score of IGFBP2 expression in stages I and II (2.02 ± 0.93) was lower than that in stages III and IV (2.98 ± 0.87) ($P = 0.0027$) (Table 3).

No significant difference was observed in the distribution of immunohistochemical staining scores of IGFBP2 expression, gender of patients and location of the tumor ($P = 0.235$) (Table 3).

Correlation between expressions of IGFBP2 and Ki-67

The expression of Ki-67 was localized to the nuclei of neoplastic cells (Figure 4). As shown in Figure 5, a significant positive correlation was observed between the

Table 3 Summary of quantitative score of IGFBP2 expression in gastric carcinoma according to clinical and pathological parameters (mean \pm SD)

Parameter	Quantitative score of IGFBP2 expression	<i>P</i>
Gender		
Male	2.05 \pm 0.82	> 0.05
Female	2.35 \pm 0.81	
Tumor site		
Cardia	2.19 \pm 0.98	> 0.05
Fundus/body	2.31 \pm 0.89	
Antrum	2.18 \pm 0.95	
Histological type		
Differentiated	2.31 \pm 0.82	> 0.05
Undifferentiated	2.72 \pm 0.88	
Lymph node metastasis		
Positive	2.16 \pm 0.98	< 0.01
Negative	2.81 \pm 0.91	
pTNM stage		
I - II	2.02 \pm 0.93	< 0.01
III - IV	2.98 \pm 0.87	
Depth wall penetration		
T1-T2	2.08 \pm 0.97	< 0.05
T3-T4	2.79 \pm 0.84	

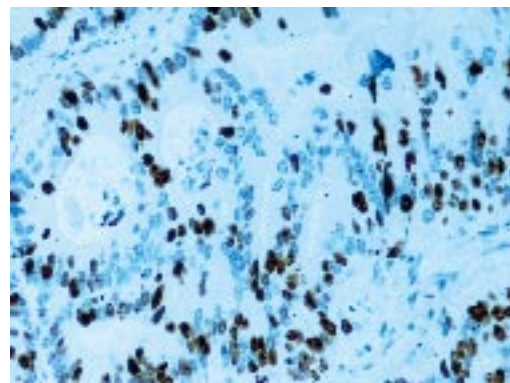


Figure 4 Ki-67 expression showing a strong nuclear staining in the tumor cells ($\times 200$).

immunohistochemical staining score of IGFBP2 and the proliferating index of Ki-67 ($P = 0.005$).

DISCUSSION

In this study, we determined the expression of IGFBP2 in 118 specimens of gastric carcinoma. To our knowledge, the expression of IGFBP2 in gastric carcinoma has not been evaluated by immunohistochemistry. Normal gastric epithelial tissue was negative or weakly positive and localized to the cytoplasm of gastric mucosa. Weak, moderate to strong expression of IGFBP2 was observed in gastric carcinoma with diffuse granular staining or strong pancytoplasmic staining. This finding is in accordance with previous reports showing that IGFBP2 can augment IGF1-induced mitogenicity of cancer cells^[14].

Serum IGFBP-2 concentrations in combination with carcinoembryonic antigen determinations are highly

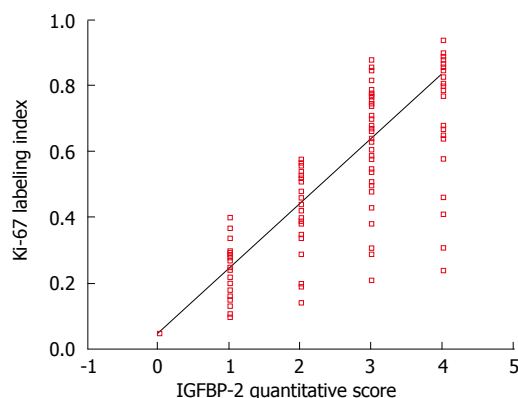


Figure 5 The positive correlation between IGFBP2 expression and Ki-67 in gastric cancer (Spearman's correlation coefficient, $P = 0.005$).

prognostic for metastasis and recurrence of colon cancers, and their use in cancer surveillance is suggested^[15,16]. In ovarian cancer patients, serum IGFBP-2 levels are positively correlated with the ovarian tumor marker CA 125^[17]. Some studies showed that serum IGFBP-2 levels in patients with prostate carcinoma are elevated and significantly correlated with PSA levels, suggesting that serum IGFBP-2 measurement in patients with low serum PSA levels can be used to monitor prostate tumor burden^[18-20]. IGFBP-2 is consistently elevated in cerebrospinal fluid (CSF) of patients with malignant tumors of the central nervous system (CNS), whereas in patients with peripheral tumors, normal IGFBP-2 levels have been found in CSF^[21], suggesting that IGFBP-2 in CSF can be considered a specific marker for CNS tumors. Busund *et al*^[13] reported that IGFBP-2 is not significantly expressed in normal and hyperplasia glandular cells of breast. Atypical hyperplasia slightly increases cytoplasmic expression of IGFBP-2, and carcinoma *in situ* shows a distinctive membrane and cytoplasmic expression. Infiltrating carcinomas strongly express cytoplasmic IGFBP-2, suggesting that IGFBP-2 might be an independent indicator of malignancy. Richardsen *et al*^[22] have found a significant over-expression of IGFBP-2 in prostatic intraepithelial neoplasia (PIN) and in more than 90% of cancers regardless of their grade. All cases of benign prostatic hyperplasia (BPH) are almost negatively or very weakly stained. In the present study, the immunohistochemical staining score of IGFBP-2 in gastric carcinomas was higher than that in normal mucosa, suggesting that over-expression of IGFBP-2 may have a stimulatory role in the pathogenesis of gastric cancer.

IGFBP-2, the second most abundant IGFBP in circulation, has traditionally been thought as a growth inhibitor. In transgenic mouse models, for example, IGFBP-2 over-expression through the CMV-promoter results in significantly reduced weight gain, presumably through IGFBP-2 sequestering of IGFs by reducing the bioavailability of these growth factors for their receptors^[23,24]. Over-expression of IGFBP-2 in human embryonic kidney fibroblasts inhibits cell proliferation, which can be reversed by the addition of exogenous IGFs, suggesting that IGFBP-2 has an inhibitory effect on IGF action^[25]. Conversely, expression of IGFBP-2 has also

been found to be positively correlated with tumor grade, including hepatoblastoma, mammary and ovarian cancer, Wilm's and adrenal tumors as well as prostate cancer^[26], indicating that IGFBP-2 might have cancer-promoting properties^[8]. Recently, Song *et al*^[27] used a yeast two-hybrid system to identify a binding partner of IGFBP-2, which was named invasion inhibitory protein 45 (Iip45), and found that IGFBP-2 could stimulate glioblastoma multiform (GBM) cell invasion, but Iip45 inhibits GBM cell invasion. Because IGFBP-2 carries the Tg domain and the RGD motif, IGFBP-2 may stimulate cell invasion via the integrin-mediated signaling pathway and Iip45 may inhibit IGFBP-2-stimulated invasion by interacting with the Tg-RGD region of IGFBP-2. Indeed, IGFBP2 has been recently found to interact with the alpha v beta 3 and alpha 5 beta 1 integrins^[28,29]. In addition, IGFBP2 can stimulate the growth of prostate cancer cells, an effect that can be blocked by MAP kinase and PI3-kinase inhibitors^[26]. IGFBP2 is co-expressed with the vascular endothelial growth factor in pseudopalisading glioma cells surrounding tumor necrosis^[30]. These findings suggest that IGFBP2 plays a key role in human cancer development.

We examined the correlation between the immunohistochemical staining score of IGFBP-2 and several clinicopathological factors. The immunostaining score of IGFBP-2 in carcinoma with T3-T4 penetration was higher than that with T1-T2 penetration. Higher immunohistochemical staining score of IGFBP-2 significantly correlated with lymph node metastasis and clinical stage. These findings suggest that IGFBP-2 might be a marker of invasive gastric carcinoma. However, in contrast to other tumors, no relationship was found between the immunohistochemical staining score of IGFBP-2 in gastric carcinoma and tumor grade. This discrepancy might be possibly attributed to the different materials studied.

Hormone status as measured by estrogen receptor (ER) and progesterone receptor (PR) correlates with IGFBP-2 in breast carcinoma^[13]. However, the relationship between proliferation and IGFBP-2 expression in various tumors including gastric carcinoma has not been analyzed. Therefore, we carried out the present study to investigate the association of proliferative activity with IGFBP-2 expression in gastric carcinoma. Cell proliferative activity is important for evaluating tumor growth. Monoclonal antibodies that recognize Ki-67 can label all proliferating cells in G1, S, G2 and M phases of the cell cycle and have been widely used to estimate the proliferative fraction of neoplasms^[31]. Our results reveal that the immunohistochemical staining score of IGFBP-2 is positively correlated with KI. These findings suggest that IGFBP-2 might play an important role in the progression of gastric carcinoma by promoting cell proliferative activity.

In conclusion, increased IGFBP-2 expression is related to malignant transformation of gastric carcinoma and plays an important role in the progression of gastric carcinoma. Although our data provide evidence for a role of IGFBP-2 in predicting biologic behavior in a relatively large series of gastric carcinoma, further studies are needed to establish the relationship between IGFBP-2 expression in this tumor and survival of patients with this tumor.

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LIVER CANCER

Prospective study of differential diagnosis of hepatic tumors by pattern-based classification of contrast-enhanced sonography

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Abstract

AIM: To prospectively evaluate the usefulness of a pattern-based classification of contrast-enhanced sonographic findings for differential diagnosis of hepatic tumors.

METHODS: We evaluated the enhancement pattern of the contrast-enhanced sonography images in 586 patients with 586 hepatic lesions, consisting of 383 hepatocellular carcinomas, 89 metastases, and 114 hemangiomas. After injecting a galactose-palmitic acid contrast agent, lesions were scanned by contrast-enhanced harmonic gray-scale sonography in three phases: arterial, portal, and late. The enhancement patterns of the initial 303 lesions were classified retrospectively, and multiple logistic regression analysis was used to identify enhancement patterns that allowed differentiation between hepatic tumors. We then used the pattern-based classification of enhancement we had retrospectively devised to prospectively diagnose 283 liver tumors.

RESULTS: Seven enhancement patterns were found to be significant predictors of different hepatic tumors. The presence of homogeneous or heterogeneous enhancement both in the arterial and portal phase was the typical enhancement pattern for hepatocellular carcinoma, while the presence of peritumoral vessels in the arterial phase and ring enhancement or a perfusion defect in the portal phase was the typical enhancement pattern for metastases, and the presence of peripheral nodular enhancement both in the arterial and portal phase was the typical enhancement pattern for

hemangioma. The sensitivity, specificity, and accuracy of prospective diagnosis based on the combinations of enhancement patterns, respectively, were 93.2%, 96.2%, and 94.0% for hepatocellular carcinoma, 87.9%, 99.6%, and 98.2% for metastasis, and 95.6%, 94.1%, and 94.3% for hemangioma.

CONCLUSION: The pattern-based classification of the contrast-enhanced sonographic findings is useful for differentiating among hepatic tumors.

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Key words: Contrast-enhanced sonography; Hepatocellular carcinoma; Metastasis; Hemangioma

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INTRODUCTION

Contrast-enhanced harmonic gray-scale sonography is a useful tool for evaluating the vascularity of liver tumors^[1-11], because it allows visualization of the blood perfusion of liver tumors without motion artifacts^[12] and it is simple, easy, and sufficiently non-invasive to be performed on an out-patient basis. In addition, it can be used in renal failure patients and patients who are allergic to iodine contrast agents.

Several investigators have reported that contrast-enhanced harmonic gray-scale sonography is a useful modality for differentiating among the types of hepatic tumors. However, no statistical evidence was presented, because they did not use multivariate logistic regression analysis for differential diagnosis of liver tumors^[13-22]. We previously classified the contrast-enhanced harmonic gray-scale sonographic findings in hepatic tumors into combinations of enhancement patterns^[23]. The results of a multiple logistic regression analysis and positive

predictive values calculated from the results of pattern combinations for each hepatic lesion demonstrated that the enhancement pattern-based classification of contrast-enhanced harmonic gray-scale sonographic findings is useful for making the differential diagnosis of hepatic tumors. However, this classification was not applied to diagnosis prospectively, and a prospective study of the enhancement pattern-based classification was needed to confirm its accuracy for the differential diagnosis for hepatic tumors. Moreover, the perfusion images in our previous study could only be obtained at a slow frame rate, because sufficient time is needed to allow the contrast agent to perfuse the tumor.

In November 2001, we began to use the newly developed contrast-enhanced harmonic gray-scale sonography mode to evaluate tumor vessels and tumor enhancement of hepatic mass lesions. Improvements in spatial and contrast resolution have now made it possible to evaluate the viability of hepatocellular carcinoma at a higher frame rate (7 frames per second) than with the previous mode^[23]. Although both the new and previous modes are based on phase inversion technology, by deliberately adjusting the time interval between the transmit pulses, the new contrast mode detects flow motion as well as bubble disruption. This allows the new mode to detect a sufficient flow signal even when there is little contrast agent left in the blood stream. As a result, the new contrast mode enabled identification of hypervascular liver tumors, e.g., advanced hepatocellular carcinoma (HCC) lesions, as hypervascular enhancement in the arterial phase at a high frame rate^[11].

In the present study, we first retrospectively classified enhancement pattern combinations for each hepatic lesion obtained with the new contrast mode described above and then prospectively diagnosed each hepatic tumor according to the pattern-based classification of enhancement we had retrospectively devised.

MATERIALS AND METHODS

Patients and tumors in the retrospective evaluation

Between November 2001 and August 2003, we examined 315 consecutive patients with hepatic tumors by conventional sonography. All patients were examined by both dual-phase helical CT and contrast-enhanced harmonic gray-scale sonography, and the patients who were suspected of having HCC were examined by arteriography. Twelve patients with 12 lesions located 12 cm or more beneath the skin surface were excluded, because the increase in attenuation of the ultrasound beam with depth made it difficult to destroy the Levovist bubbles in such lesions^[23]. Thus, the remaining 303 patients (238 with solitary focal lesions and 65 with multiple focal lesions) were enrolled in this study, and 303 hepatic lesions were evaluated the largest lesion in patients with more than one focal lesion. Since no significant difference in sensitivity calculated from the results of pattern combinations for each hepatic lesion according to the contrast-enhanced harmonic gray-scale sonographic findings was observed between solitary lesions and multiple lesions in the previous study^[23], we enrolled the

patients with both solitary and multiple lesions. There were 173 men (104 HCC, 38 metastases, 31 hemangiomas) and 130 women (74 HCC, 18 metastases, 38 hemangiomas), without significant difference in age between the men (65.2 ± 9.6 years) and women (64.4 ± 11.2 years). We classified cholangiocellular carcinoma as hepatic metastasis because pathologically the tumors are adenocarcinomas originating from the intrahepatic bile duct.

The final diagnosis of the lesions studied was HCC in 178 patients, liver metastasis in 56 patients (14 from colon carcinoma, 11 from pancreatic carcinoma, 7 from rectal carcinoma, 6 from cholangiocellular carcinoma, 3 from gastric carcinoma, 3 from gallbladder carcinoma, 2 from malignant melanoma, 2 from lung carcinoma, and 1 each from malignant lymphoma, nasopharyngeal carcinoma, esophageal carcinoma, laryngeal carcinoma, thyroid carcinoma, and hemangiopericytoma), and hemangioma in 69 patients. All HCCs were histologically diagnosed after surgical resection (12 lesions), sonography-guided biopsy (162 lesions), or autopsy (4 lesions). All liver metastases were histologically diagnosed after surgical resection (6 lesions), sonography-guided biopsy (48 lesions), and autopsy (2 lesions). The diagnosis of hemangioma was confirmed by contrast-enhanced CT and MR and the absence of any changes on follow-up images more than 1 year later. Of the 178 patients with HCC lesions, 155 had cirrhosis, and the diagnosis was made histologically and/or clinically.

The hepatic tumors were measured on conventional US images by one of the two operators who performed the contrast-enhanced harmonic imaging. The mean maximal diameters were: 26 ± 16 mm for HCCs, 33 ± 21 mm for metastases, and 28 ± 19 mm for hemangiomas. The numbers of HCC lesions, classified according to maximal tumor diameter, were 2 lesions < 10 mm, 87 lesions between 10 mm and 20 mm, and 89 lesions > 20 mm.

Patients and tumors in the prospective evaluation

Between September 2003 and April 2005, we examined 283 consecutive patients (195 men with age 67.1 ± 9.2 years, and 88 women with age 68.0 ± 8.8 years) with hepatic tumors by conventional US, and using the pattern-based classification of enhancement, we retrospectively devised to prospectively diagnose their liver tumors. Solitary focal lesions were detected in 207 patients and multiple focal lesions in 76 patients.

The final diagnosis of the lesions was HCC in 205 patients, liver metastasis in 33 patients (8 from colon carcinoma, 8 cholangiocellular carcinoma, 4 from pancreatic carcinoma, 4 from gastric carcinoma, 2 from gallbladder carcinoma and 1 each from esophageal carcinoma, lung carcinoma, rectal carcinoma, prostate carcinoma, malignant lymphoma, adenoid cystic carcinoma, and leiomyosarcoma), and hemangioma in 45. All liver metastases were histologically diagnosed after surgical resection (5 lesions), sonography-guided biopsy (27 lesions), and autopsy (1 lesion). Of the 205 HCC lesions, 12 were histologically diagnosed after resection, 194 after sonography-guided biopsy, and the remaining 3 after autopsy. Of the 205 patients with HCC lesions, 177 had cirrhosis which was diagnosed histologically and/or

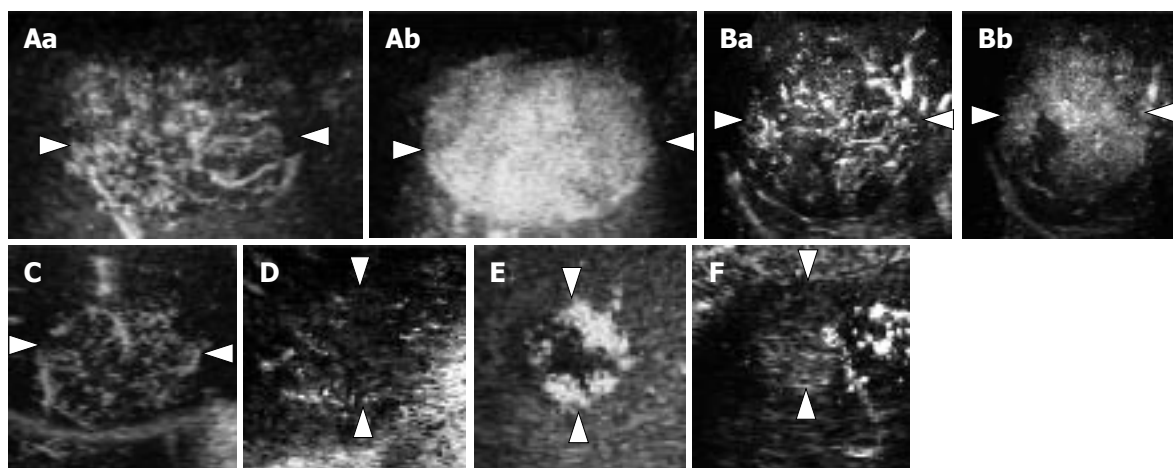


Figure 1 Diagram showing enhancement patterns of hepatic tumors in the arterial phase. **A:** Intratumoral vessels in the early arterial phase (a) with homogeneous enhancement in the late arterial phase (b) (pattern A1); **B:** Intratumoral vessels in the early arterial phase (a) with heterogeneous enhancement in the late arterial phase (b) (pattern A1); **C:** Intratumoral vessels without homogeneous or heterogeneous enhancement (pattern A2); **D:** Peritumoral vessels alone (pattern A3); **E:** Peripheral nodular enhancement without tumor vessels (pattern A4); **F:** No enhancement and no tumor vessels (pattern A5).

clinically.

The hepatic tumors were measured by conventional US by one of the two operators who performed the contrast-enhanced harmonic imaging. The mean maximal diameter of the HCCs was 26 ± 15 mm, of the metastases was 33 ± 17 mm, and of the hemangiomas was 30 ± 22 mm.

Informed consent was obtained from all patients before the study, and the study was approved by Institutional Ethics Committee.

We defined “early HCC lesions” as well-differentiated cancers with no substantial destruction of the preexisting hepatic framework^[24].

Procedures

To minimize variations between operators, the contrast-enhanced harmonic gray-scale imaging studies were performed by either one of two operators (K. N., T. I.) using the same examination protocol. Neither operator was aware of the results of the helical CT and angiography examinations or the histological diagnosis. Contrast-enhanced harmonic gray-scale sonography was performed with a SONOLINE Elegra machine (Siemens Medical Systems, Issaquah, WA), a 3.5-MHz convex probe, and Sie flow mode imaging software until the end of September 2004, and with a LOGIQ 7 machine (GE Healthcare, Milwaukee, WI), a 3.5-MHz convex probe, and Coded harmonic angio mode imaging software from October 2004 onward.

After intravenous bolus injection of a half vial of the 300 mg/mL concentration of galactose–palmitic acid mixture contrast medium (Levovist; Schering AG, Berlin, Germany), the liver was scanned by real-time contrast-enhanced harmonic gray-scale sonography at 5–13 frames per second, usually at 7 frames per second. The transmission power was 100%, and the mechanical index values were between 1.0 and 1.6. The focus position was just below the bottom of the tumor. Levovist is a suspension of galactose (99.9%) stabilized with 0.1% palmitic acid. A 3.5-mL dose of this agent was injected at

0.5 mL/s via a 22-gauge cannula placed in an antecubital vein. After the bolus injection of Levovist, 50 g/L glucose was continuously infused at 5 mL/min. The patients gently inspired and then held their breath for about 30 s (10–40 s after the contrast medium injection) while the tumor vessels and tumor enhancement were examined (arterial phase).

After observation of the arterial phase, we froze the image. In the cases scanned with the Elegra machine, we reviewed the images frame by frame from cine loop memories and stored them on magneto-optical disks. In the cases scanned with the LOGIQ 7 machine, we stored the images as a cine clip with GE exclusive raw-data format files in the LOGIQ 7 computer. This procedure took approximately 15–35 s (mean, 25 s), and we used the time to allow the contrast agent to pool in the hepatic parenchyma. We then scanned the whole tumor and examined the tumor for enhancement 60–120 s after injection of the contrast agent while the patients held their breath for a few seconds (portal phase). We froze the images and stored them by the same method as mentioned above. Finally, 5 min after injection of the contrast agent, we examined the lesion in a sweep scan to determine if it was iso-echoic or hypo-echoic (late phase). We stored these images by the same method as described above. The complete examinations were recorded on S-VHS videotape.

Image evaluation

We evaluated the images for the presence and shape of tumor vessels and the enhancement patterns during the arterial phase and classified the patterns into five categories (Figure 1) as follows: pattern A1, intratumoral vessels with homogeneous (Figure 1A) or heterogeneous enhancement (Figure 1B); pattern A2, intratumoral vessels without homogeneous or heterogeneous enhancement (Figure 1C); pattern A3, peritumoral vessels alone (Figure 1D); pattern A4, peripheral nodular enhancement without tumor vessels (Figure 1E); and pattern A5, no enhancement

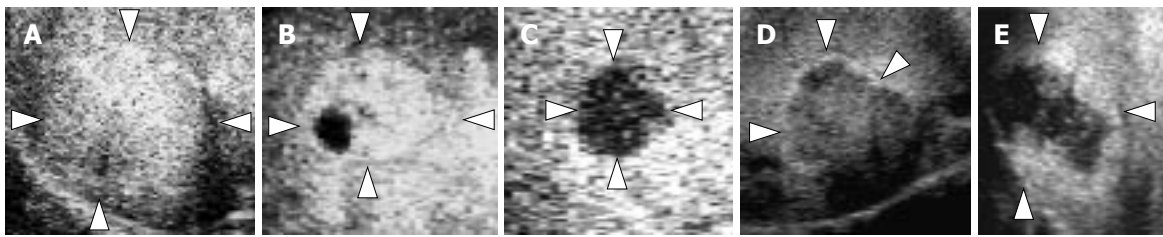


Figure 2 Diagram showing enhancement patterns of hepatic tumors in the portal phase. **A:** Homogeneous enhancement (pattern P1); **B:** Heterogeneous enhancement (pattern P1); **C:** Perfusion defect (pattern P2); **D:** Ring enhancement (pattern P3); **E:** Peripheral nodular enhancement (pattern P4).

and no tumor vessels (Figure 1F). In the portal phase, the enhancement patterns of the lesions were classified into four categories (Figure 2) as follows: pattern P1, homogeneous (Figure 2A) or heterogeneous enhancement (Figure 2B); pattern P2, perfusion defect (Figure 2C); pattern P3, ring enhancement (Figure 2D); and pattern P4, peripheral nodular enhancement (Figure 2E). The enhancement patterns of the lesion in the late phase were classified into two categories relative to the enhancement pattern in the surrounding liver parenchyma (Figure 3): pattern L1, iso-echoic (Figure 3A); and pattern L2, hypo-echoic (Figure 3B).

The image evaluation was performed independently by two readers (K.T., M.M.), both of whom reviewed all of the sonographic images recorded on video-tape, cine clips, and magneto-optical disks, and they were asked to classify each lesion into one of the patterns shown in Figures 1-3. The readers had no knowledge of the results of the helical CT or angiography, or of the histological diagnosis. Since 16 of the 586 hepatic lesions were classified differently by the two readers, the two readers and the two operators had a consensus meeting to arrive at a consensus for the final classification.

Statistical analysis

A multiple logistic regression analysis was performed to select independent variables of imaging features associated with the dependent variable, i.e., tumor type, as described previously^[23]. Since only dichotomous variables can be used as the dependent variable in multiple logistic regression analyses, we used one of the three tumor types *versus* the other two tumor types as the dependent variables in our analysis. The independent variables were different imaging features observed in the three different phases after contrast enhancement, and each variable had a dichotomous value (not observed = 0, observed = 1). We selected independent variables with a *P* value less than 0.05 in the multiple logistic regression analysis and used these significant independent variables to construct a multivariable model. All tumors exhibited one of the defined combinations of statistically significant independent variables, and statistically significant independent variables were used to make the defined combinations. The diagnostic performance of our multivariable model was evaluated by calculating the positive predictive values according to Bayes theorem^[25], based on sensitivity, specificity, and prior probability (positive predictive values = prior probability × sensitivity/

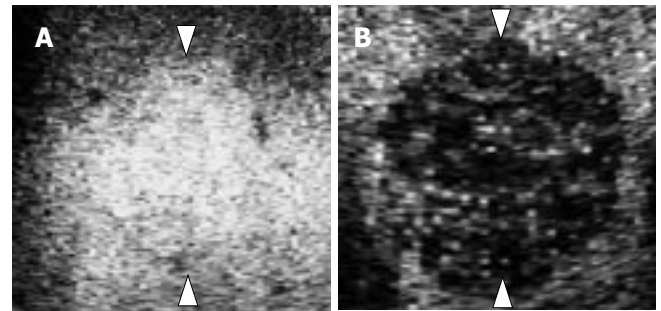


Figure 3 Diagram showing enhancement patterns of hepatic tumors in the late phase. **A:** Iso-echoic (pattern L1); **B:** Hypo-echoic (pattern L2).

{sensitivity × prior probability + (1-specificity) × (1- prior probability)}). Prior probability was calculated by dividing the numbers of HCCs, metastases, and hemangiomas by the total number of tumors (303 tumors). The diagnosis of each hepatic tumor was made on the basis of the largest positive predictive value of each combined enhancement pattern based on the results of the retrospective study, and the enhancement patterns of the prospective study were used to make the diagnosis based on the results of the retrospective study. Finally, we calculated the sensitivity, specificity, and accuracy of each tumor diagnosis based on the results of the enhancement pattern-based classification system as described above. The SPSS computer program (SPSS Japan, Tokyo, Japan) was used to perform the data analysis.

RESULTS

Retrospective study

Arterial phase: Table 1 shows the enhancement patterns of hepatic tumors observed by contrast-enhanced harmonic gray-scale sonography. In the arterial phase, 169 (95%) of the 178 HCC lesions showed intratumoral vessels with homogeneous or heterogeneous enhancement (pattern A1), and among the other 9 HCC lesions, 1 (1%) showed intratumoral vessels without homogeneous or heterogeneous enhancement (pattern A2), 2 (1%) showed a peripheral nodular enhancement without tumor vessels (pattern A4), and 6 (3%) showed no enhancement and no tumor vessels (pattern A5). Five (9%) of the 56 liver metastases showed intratumoral vessels with homogeneous or heterogeneous enhancement (pattern A1), 15 (27%) showed intratumoral vessels without

Table 1 Retrospective study of enhancement patterns of hepatic tumors

	Hepatocellular carcinoma <i>n</i> = 178	Metastasis <i>n</i> = 56	Hemangioma <i>n</i> = 69
Arterial phase			
A1	95% (169/178)	9% (5/56)	0% (0/69)
A2	1% (1/178)	27% (15/56)	1% (1/69)
A3	0% (0/178)	55% (31/56)	0% (0/69)
A4	1% (2/178)	0% (0/56)	61% (42/69)
A5	3% (6/178)	9% (5/56)	38% (26/69)
Portal phase			
P1	93% (166/178)	16% (9/56)	46% (32/69)
P2	4% (8/178)	14% (8/56)	0% (0/69)
P3	1% (2/178)	66% (37/56)	1% (1/69)
P4	1% (2/178)	4% (2/56)	52% (36/69)
Late Phase			
L1	8% (14/178)	0% (0/56)	20% (14/69)
L2	92% (164/178)	100% (56/56)	80% (55/69)

A1: Intratumoral vessels with homogeneous or heterogeneous enhancement; A2: Intratumoral vessels without homogeneous or heterogeneous enhancement; A3: Peritumoral vessels; A4: Peripheral nodular enhancement without tumor vessels; A5: No enhancement and no tumor vessels; P1: Homogeneous or heterogeneous enhancement; P2: Perfusion defect; P3: Ring enhancement; P4: Peripheral nodular enhancement; L1: Iso-echoic; L2: Hypo-echoic.

homogeneous or heterogeneous enhancement (pattern A2), 31 (55%) showed peritumoral vessels (pattern A3), and 5 (9%) showed no enhancement and no tumor vessels (pattern A5). One (1%) of the 69 hemangiomas showed intratumoral vessels without homogeneous or heterogeneous enhancement (pattern A2), 42 showed peripheral nodular enhancement without tumor vessels in the arterial phase (pattern A4), and 26 (38%) showed no enhancement and no tumor vessels (pattern A5).

Portal phase: In the portal phase, 166 (93%) of the 178 HCC lesions showed a homogeneous or heterogeneous pattern of enhancement (pattern P1): a homogeneous pattern in 148 (83%), and a heterogeneous pattern in 18 (10%). Since Levovist did not enhance the necrotic areas of the tumors, tumors that contained necrotic areas showed a heterogeneous pattern^[23]. Eight (4%) of the 178 lesions showed a perfusion defect in the portal phase (pattern P2): two exhibited arteriportal shunting on arteriography and intratumoral vessels with homogeneous enhancement in the arterial phase, and the remaining 6 lesions were early HCCs. Two (1%) of the remaining 4 (2%) HCC lesions showed ring enhancement (pattern P3) and 2 (1%) showed a peripheral nodular enhancement (pattern P4). Nine (16%) of the 56 liver metastases showed homogeneous or heterogeneous enhancement (pattern P1), 8 (14%) showed a perfusion defect (pattern P2), 37 (66%) showed ring enhancement (pattern P3), and 2 (4%) showed peripheral nodular enhancement (pattern P4). Of the 69 hemangiomas, 32 (46%) showed homogeneous enhancement (pattern P1), 1 (1%) showed ring enhancement (pattern P3), and 36 (52%) showed peripheral nodular enhancement (pattern P4).

Late phase: In the late phase, 164 (92%) of the

Table 2 Logistic regression analyses for the diagnosis of hepatocellular carcinoma, metastasis, and hemangioma

	Odds ratio	95% CI	<i>P</i>
Hepatocellular carcinoma			
A1	189.665	25.716-1398.871	< 0.01
P1	38.993	4.616-329.381	< 0.01
Metastases			
A3	73.139	7.19-743.997	< 0.01
P2	27.973	5.202-150.421	< 0.01
P3	137.385	27.064-697.421	< 0.01
Hemangioma			
A4	102.175	15.567-670.619	< 0.01
P4	695.141	110.903-4357.157	< 0.01

A1: Intratumoral vessels with homogeneous or heterogeneous enhancement; A3: Peritumoral vessels; A4: Peripheral nodular enhancement without tumor vessels; P1: Homogeneous or heterogeneous enhancement; P2: Perfusion defect; P3: Ring enhancement; P4: Peripheral nodular enhancement.

HCC lesions were visualized as hypo-echoic with the surrounding liver parenchyma (pattern L1), and 14 (8%) as iso-echoic (pattern L2). Histological examination revealed that all iso-echoic HCCs lesions in the late phase were well differentiated HCCs. All metastases were hypo-echoic (pattern L1). Of the 69 hemangiomas, 55 (80%) were hypo-echoic (pattern L1), and the remaining 14 (20%) were iso-echoic (pattern L2).

Factors predicting the diagnosis of hepatic tumors:

To identify predictors of the diagnosis of hepatic tumors, multiple logistic regression analysis was performed on 11 parameters based on the results of contrast-enhanced harmonic gray-scale sonography with contrast agents. Only 7 parameters were selected as independent variables associated with a type of hepatic tumor (Table 2). Intratumoral vessels with homogeneous or heterogeneous enhancement in the arterial phase (pattern A1) (odds ratio: 189.665; *P* < 0.01) and homogeneous or heterogeneous enhancement in the portal phase (pattern P1) (odds ratio: 38.993; *P* < 0.01) were selected as statistically significant variables to differentiate HCC from the other two types of hepatic lesions combined, i.e., metastases and hemangiomas. Peritumoral vessels in the arterial phase (pattern A3) (odds ratio: 73.139; *P* < 0.01), perfusion defect in the portal phase (pattern P2) (odds ratio: 17.92; *P* < 0.01), and ring enhancement in the portal phase (pattern P3) (odds ratio: 137.385; *P* < 0.01) were selected as statistically significant variables to differentiate metastases from the other two types of lesions combined, i.e., HCCs and hemangiomas. Peripheral nodular enhancement without tumor vessels in the arterial phase (pattern A4) (odds ratio: 102.175; *P* < 0.01) and peripheral nodular enhancement in the portal phase (pattern P4) (odds ratio: 695.141; *P* < 0.01) were selected as statistically significant variables to differentiate hemangiomas from the other two types of lesions combined, i.e., HCCs and metastases. Two parameters of the late phase were not statistically significant variables.

Enhancement pattern-based classification: Combinations of patterns of enhancement in the arterial phase (A1, A3, A4, and others) and portal phase (P1, P2, P3, and

Table 3 Enhancement pattern-based classification of contrast-enhanced harmonic gray-scale sonography images and positive predictive value for differentiating hepatic tumors

Enhancement pattern Pattern combi- nation	Arterial phase		Positive predictive value (No. of lesions)			Diagnosis ¹
	Arterial phase	Portal phase	HCC (n = 178)	Metastasis (n = 56)	Hemangioma (n = 69)	
1	A1	P1	0.996 (165)	0.004 (2)	0 (0)	HCC
2	A1	P2	1 (2)	0 (0)	0 (0)	HCC
3	A1	P3	0 (0)	1 (3)	0 (0)	Metastasis
4	A1	P4	1 (2)	0 (0)	0 (0)	HCC
5	A3	P1	0 (0)	1 (4)	0 (0)	Metastasis
6	A3	P2	0 (0)	1 (3)	0 (0)	Metastasis
7	A3	P3	0 (0)	1 (24)	0 (0)	Metastasis
8	A4	P1	0 (0)	0 (0)	1 (25)	Hemangioma
9	A4	P3	0.84 (2)	0 (0)	0.16 (1)	HCC
10	A4	P4	0 (0)	0 (0)	1 (16)	Hemangioma
11	others ²	P1	0.20 (1)	0.19 (3)	0.61 (8)	Hemangioma
12	others ²	P2	0.79 (6)	0.21 (5)	0 (0)	HCC
13	others ²	P3	0 (0)	1 (11)	0 (0)	Metastasis
14	others ²	P4	0 (0)	0.04 (1)	0.96 (19)	Hemangioma

A1: Intratumoral vessels with homogeneous or heterogeneous enhancement; A3: Peritumoral vessels; A4: Peripheral nodular enhancement without tumor vessels; P1: homogeneous or heterogeneous enhancement; P2: Perfusion defect; P3: Ring enhancement; P4: Peripheral nodular enhancement. ¹Diagnosis was made on the basis of the largest positive predictive value for each of the three kinds of tumors in each combination of enhancement patterns. ²Others mean A2 or A5 as shown in Table 1.

P4) were then analyzed, and 16 different combinations of statistically significant predictors in the arterial phase and portal phase were identified. Since two combinations of patterns were not observed in any of the cases in this study, only 14 patterns were analyzed (Table 3).

Enhancement pattern combinations 1, 2, 4, 9, and 12 showed high positive predictive value for HCC, and “pattern combination 1” was the most predominant pattern among them. According to the greatest tumor diameter, the numbers of all lesions that were “pattern combination 1” lesions were: 2 (100%) of 2 lesions less than 10 mm; 82 (94%) of 87 lesions between 10 and 20 mm; and 81 (91%) of 89 lesions greater than 20 mm. Both “pattern combination 2” lesions were HCCs with arteriportal shunting. Five of the six “pattern combination 12” lesions were early HCCs.

The enhancement pattern combinations 3, 5, 6, 7, and 13 showed high positive predictive value for metastasis, and “pattern combination 7” was the predominant pattern. “Pattern combinations 3 and 13” were observed in hypervascular metastases.

Enhancement “pattern combinations 8, 10, 11, and 14” showed high positive predictive value for hemangioma, and “pattern combination 8” was the most predominant pattern among them. The hemangiomas that showed “pattern combination 8” were relatively high-flow type hemangiomas. All 8 lesions that showed “pattern combination 11” exhibited no enhancement and no tumor vessels in the arterial phase, and homogeneous enhancement in the portal phase.

Table 4 Prospective study of pattern combination-based classification of contrast-enhanced harmonic gray-scale sonography images for differentiating hepatic tumors

Enhancement pattern Pattern combi- nation	Arterial phase		No. of lesions			Diagnosis ¹
	Arterial phase	Portal phase	HCC (n = 205)	Metastasis (n = 33)	Hemangioma (n = 45) ²	
1	A1	P1	183	3	0	HCC
2	A1	P2	0	0	0	HCC
3	A1	P3	0	5	0	Metastasis
4	A1	P4	0	0	0	HCC
5	A3	P1	0	1	1	Metastasis
6	A3	P2	0	1	0	Metastasis
7	A3	P3	0	14	0	Metastasis
8	A4	P1	0	0	16	Hemangioma
9	A4	P3	0	0	0	HCC
10	A4	P4	0	0	7	Hemangioma
11	Others ³	P1	14	1	2	Hemangioma
12	Others ³	P2	8	0	0	HCC
13	Others ³	P3	0	8	0	Metastasis
14	Others ³	P4	0	0	18	Hemangioma

A1: Intratumoral vessels with homogeneous or heterogeneous enhancement; A3: Peritumoral vessels; A4: Peripheral nodular enhancement without tumor vessels; P1: Homogeneous or heterogeneous enhancement; P2: Perfusion defect; P3: Ring enhancement; P4: Peripheral nodular enhancement. ¹Diagnosis was made on the basis of the largest positive predictive value for each of the three kinds of tumors in each combination of enhancement patterns in the retrospective study. ²One case of hemangioma was not diagnosed because the pattern combination did not exist in Table 3. ³Others mean A2 or A5 as shown in Table 1.

Prospective study

Table 4 shows the results of a prospective study of hepatic tumors diagnosed by using the pattern combination-based classification of the contrast-enhanced harmonic gray-scale sonography findings. Three combination patterns were not found in any of the cases in the prospective study.

Of the 205 HCCs, 183 lesions corresponded to “pattern combination 1”, and 8 corresponded to “pattern combination 12”; these lesions were correctly diagnosed as HCC. According to greatest tumor diameter, the numbers of all lesions that were “pattern combination 1” were: 1 (50%) of 2 lesions less than 10 mm; 80 (83%) of 96 lesions between 10 mm and 20 mm; and 102 (95%) of 107 lesions greater than 20 mm. Fourteen HCC lesions were not diagnosed as HCC. These lesions were corresponded to “pattern combination 11” and were diagnosed as hemangioma. Eleven of the 14 “pattern combination 11” lesions showed intratumoral vessels without homogeneous or heterogeneous enhancement in the arterial phase (pattern A2), and the remaining three showed no enhancement and no tumor vessels in the arterial phase (pattern A5). These lesions were histologically diagnosed as well differentiated HCC.

Of the 33 metastases, 14 corresponded to “pattern combination 7”, 8 to “pattern combination 13”, 5 to “pattern combination 3”, and one each to “pattern combinations 5 and 6”. These 29 lesions were correctly diagnosed as metastases. Four metastases were not diagnosed as metastases; three corresponding to “pattern

combination 1" were diagnosed as HCC, and one corresponding to "pattern combination 11" was diagnosed as hemangioma.

Of the 45 hemangiomas, 16 corresponded to "pattern combination 8", 7 to "pattern combination 10", 18 to "pattern combination 14", and 2 to "pattern combination 11". These hemangioma lesions were not correctly diagnosed as hemangiomas. One hemangioma was not diagnosed as hemangioma; it corresponded to "pattern combination 5" and was diagnosed as a metastasis. The remaining one case of hemangioma was not diagnosed because the pattern combination did not exist in the retrospective study.

The sensitivity, specificity, and accuracy of prospective diagnosis based on the combinations of enhancement patterns, respectively, were 93.2%, 96.2%, and 94.0% for hepatocellular carcinoma, 87.9%, 99.6%, and 98.2% for metastasis, and 95.6%, 94.1%, and 94.3% for hemangioma.

DISCUSSION

In the present study, we first retrospectively classified the contrast-enhanced harmonic gray-scale sonography findings in hepatic tumors into combinations of enhancement patterns. The results of a multiple logistic regression analysis and the positive predictive values calculated from the results of the pattern combinations for each hepatic lesion demonstrated that the enhancement pattern-based classification of contrast-enhanced harmonic gray-scale sonography findings was useful for making the differential diagnosis of hepatic tumors in the subsequent prospective study. Hence, we concluded that contrast-enhanced harmonic gray-scale sonography is a useful modality for differentiating among the types of hepatic tumors we studied.

To visualize tumor perfusion in our previous mode of contrast-enhanced harmonic gray-scale sonography, we reduced bubble destruction and used a frame rate of 2 frames per second, and at that rate intratumoral vessels were observed in the arterial phase in 98 (84%) of the 116 lesions^[23]. We used the novel mode in the present study and 169 (95%) of the 178 hepatocellular carcinoma lesions showed homogeneous or heterogeneous enhancement with intratumoral vessels in the arterial phase. Clearer and more frequent visualizations of homogeneous or heterogeneous enhancement with intratumoral vessels were achieved when the frame rate was 7 frames per second. This real-time observation of the arterial phase made the diagnosis of hepatocellular carcinoma easier when the lesion showed hypervascular enhancement with intratumoral vessels. This novel mode of real-time contrast-enhanced harmonic gray-scale sonography also enabled detection of viable hepatocellular carcinoma lesions which were not detected by conventional sonography^[11], and we were able to treat these lesions in real time by percutaneous therapy under guidance by this modality. Improved sensitivity also permitted us to perform this examination after injection of a smaller volume of contrast agent than with the previous mode^[11].

The arteries feeding hepatic tumors are directly supplied by branches of the hepatic artery. Hemangiomas

are hypervascular tumors in which normal-caliber arteries taper normally and subdivide normally into small vessels^[26]. The typical finding of hemangiomas in previous mode was absence of enhancement in the arterial phase^[23]. However, the typical findings of hemangioma in the present study were peripheral nodular enhancement without tumor vessels in the arterial phase of contrast-enhanced harmonic gray-scale sonography. The novel contrast mode improved both spatial resolution and contrast resolution, and this development made it possible to observe peripheral nodular enhancement in the arterial phase. The arterial branches supplying HCCs tend to show irregularly tortuous extension, whereas the arterial branches supplying hepatic metastases are scanty or fine, and are located in the periphery of the tumors, distinguishing them from HCCs^[27]. The typical arterial phase finding in metastases is peritumoral vessels without early enhancement, whereas the typical finding in HCCs is intratumoral vessels with homogeneous or heterogeneous enhancement. These different findings in the arterial phase may be of use in differentiating between hypervascular metastasis and typical HCC. Moreover, more than 80% of metastases show ring enhancement or a perfusion defect in the portal phase, whereas more than 95% of HCCs show homogeneous or heterogeneous enhancement in the portal phase. However, the tumor vessels of hepatic metastases exhibit many variations. Hypervascular metastases sometimes show intratumoral vessels with homogeneous or heterogeneous enhancement in the arterial phase, and this enhancement pattern mimics that of HCC. In the portal phase, however, almost all hypervascular metastases showed ring enhancement. Thus, the combinations of pattern enhancement in the arterial and the portal phase are useful for making the differential diagnosis of hepatic tumors.

The multivariate analysis in the present study showed that the parameters in the late phase were not significant predictors of the diagnosis, suggesting that observation of the vascular phase (arterial and portal phase) is important in making the differential diagnosis of hepatic tumors. This finding is the same as with the previous mode^[23]. We think that the late phase may be useful for detecting metastases, because all metastases were visualized as a hypo-echoic lesion in the late phase. All HCC lesions that exhibited "pattern combination 1" in the vascular phase and that were iso-echoic in the late phase were well differentiated HCC, whereas almost all HCC lesions with "pattern combination 1" in the vascular phase and hypo-echoic in the late phase were moderately to poorly differentiated HCC. These results are similar to those reported by Nicolau *et al*^[28] in a study using SonoVue, a second generation ultrasound contrast agent. We think that the late phase finding may be useful for evaluating the grade of malignancy of HCC. However, they are not of value for differentiating hepatic tumors, because whether a lesion is iso-echoic or hypo-echoic with the surrounding liver parenchyma is not a significant predictor of the diagnoses of hepatic tumors.

In this prospective study, the pattern-based classification failed to correctly diagnose 14 HCC lesions that were histologically well differentiated HCCs. These lesions

did not show early homogeneous or heterogeneous enhancement in the arterial phase, but showed homogeneous or heterogeneous enhancement in the portal phase. These misdiagnoses are attributable to the fact that there were only a small number of well differentiated HCC lesions in the retrospective study compared to the prospective study, because 1 HCC lesion, 3 metastatic lesions, and 8 hemangioma lesions exhibited this pattern of enhancement in the retrospective study and it had high positive predictive value for hemangioma.

The European Association for the Study of the Liver (EASL) conference has stated that HCC can be diagnosed without biopsy in patients with cirrhosis who have a lesion greater than 2 cm in diameter that shows characteristic arterial vascularization on two different imaging modalities, i.e., triphasic CT scan and MRI^[29,30]. Such lesions should be treated as HCC, since the positive predictive value of the clinical and radiological findings exceeds 95%^[29,30]. In this study, 348 (93%) of the 379 HCC lesions equal to or greater than 10 mm exhibited the most predominant pattern of HCC lesions on contrast-enhanced sonography. Because contrast-enhanced sonography has highly accuracy for the diagnosis of HCC, we hope that this modality will be included in imaging modalities like triphasic CT scan and MRI.

We prospectively diagnosed hepatic tumors correctly according to the results of the enhancement pattern based on the retrospective study using Levovist, a first-generation contrast agent, suggesting that this enhancement pattern-based classification will be useful for differentiating among the hepatic tumors in future trials using second-generation contrast agents.

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Cytoplasmic expression of p27^{kip1} is associated with a favourable prognosis in colorectal cancer patients

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Abstract

AIM: To evaluate the prognostic significance of p27^{kip1} in colorectal cancer patients.

METHODS: Cytoplasmic and nuclear p27^{kip1} expression was evaluated in 418 colorectal cancers using tissue microarrays. Data were associated with known patient and tumor variables and long-term patient outcomes, providing further insight into the mechanisms by which p27^{kip1} may influence tumor development.

RESULTS: Nuclear and cytoplasmic p27^{kip1} expressions were detected in 59% and 19% of tumors respectively. Cytoplasmic p27^{kip1} was almost invariably associated with positive nuclear p27^{kip1} expression. Neither case correlated with known clinical or pathological variables, including tumor stage, grade or extramural vascular invasion. Furthermore, nuclear p27^{kip1} expression had no impact on survival. However, we identified a significant correlation between expression of cytoplasmic p27^{kip1} and longer disease-specific survival times. On multivariate analysis, TNM stage and extramural vascular invasion were highly significant independent prognostic factors, with positive cytoplasmic p27 expression showing a trend towards improved patient survival ($P = 0.059$).

CONCLUSION: These findings support the recent evidence that cytoplasmic p27^{kip1} has a distinct and important biological role that can influence tumor outcome.

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Key words: Colorectal cancer; Tissue microarray; p27^{kip1}; Prognostic factor

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INTRODUCTION

p27, also known as Kip1, is expressed in most cells and its role is to bind and inhibit cyclin/cyclin-dependent kinase (cyclin-CDK) complexes, thereby inhibiting cell cycle progression^[1]. This role in the cell cycle makes p27^{kip1} a key player in multiple cell fate decisions including proliferation, motility, differentiation and apoptosis. Numerous studies have shown that p27^{kip1} is a tumor suppressor gene whose loss co-operates with mutations in several oncogenes and tumor suppressor genes in order to facilitate tumor growth. p27^{kip1} is rarely mutated, and is predominantly regulated at the post-transcriptional level by degradation in the ubiquitin-proteasome pathway^[2]. However, in contrast to most tumor suppressor genes, which are recessive, tumor suppression by p27^{kip1} appears critically dependent upon on the absolute level of p27^{kip1}^[3]. Several clinical studies have correlated absent or low p27^{kip1} expression with a poor prognosis in a range of malignancies, including breast, colorectal, gastric, ovary, prostate, bladder and oesophageal tumors^[4-11]. Other investigators have failed to reproduce, or have only partially confirmed, the previously published results^[12-14]. Some controversy also exists over the importance of cytoplasmic expression of p27^{kip1}, which was originally thought to represent a mechanism for inactivating p27^{kip1} by sequestering it away from its site of action within the nucleus^[15]. Cytoplasmic expression of p27^{kip1} has been identified in colorectal cancer, ovarian cancer and Barrett's oesophagus^[6,16-18], and recent evidence

suggests that it may have an active role, as it has been shown to bind to RhoA and inhibit its activity^[19]. Members of the Rho family include Cdc42, Rac and RhoA. These act as molecular switches in signalling pathways affecting gene transcription and cytoskeletal rearrangements. In particular, the Rho proteins regulate and co-ordinate the cytoskeletal remodelling that underlies changes in cell adhesion and migration^[20]. Consistent with these studies, p27's ability to regulate motility is distinct from its ability to inhibit cellular proliferation. Indeed, the region of p27^{kip1} that binds to RhoA and is required for migration is different from the region that binds cyclin-CDKs^[21].

Using high-throughput Tissue Microarray (TMA) technology^[22] we have investigated the expression of both cytoplasmic and nuclear p27^{kip1} in a series of over 400 paraffin wax-embedded colorectal tumor specimens. Data derived from this analysis was then associated with known patient and tumor variables, and with long-term patient outcome data, in order to gain further insight into the mechanisms by which p27 may influence tumor development.

MATERIALS AND METHODS

Patients

Four hundred and sixty-two patients were included in this study. A detailed description of these cases has been provided previously^[23,24]. Briefly, all patients included in the TMA underwent elective resection of a histologically proven primary colorectal cancer between 1st January 1993 and 31st December 2000 at the University Hospital Nottingham, with prospective collection of associated clinical and pathological data including tumor site, TNM stage, histological tumor type and grade, and the presence of extramural vascular invasion. Histological factors more recently identified as having potential prognostic value in colorectal cancer, such as tumor budding and the tumor border configuration, were not recorded routinely at our institution and hence were not considered in the data analysis. Patients with lymph node positive disease were characteristically treated with adjuvant chemotherapy, consisting of 5-fluorouracil and folinic acid. The UK Office for National Statistics has provided comprehensive follow-up regarding the date and cause of death for this cohort of patients. The length of follow-up was determined from the date of primary tumor resection, with surviving cases censored for analysis on the 31st December 2003. Disease specific survival was used as the primary end-point of the study.

Immunohistochemical procedures

Arrayed tumors were analysed for the expression of p27^{kip1} using a murine monoclonal anti-human p27^{kip1} antibody (clone SX53G8; Dako Ltd, Ely, UK) and standard immunohistochemistry with an avidin-biotin/horseradish peroxidase development system. Five microns formalin-fixed, wax-embedded tissue array sections were dewaxed in an incubator for 20 min at 60°C, deparaffinised with xylene, rehydrated through graded alcohol and immersed in 0.3% hydrogen peroxide-methanol solution to block endogenous peroxidase activity. Antigen retrieval was

achieved by microwaving slides for 20 min in pH 9.0 EDTA buffer. Endogenous avidin/biotin activity was blocked using an avidin/biotin blocking kit (Vector Labs, USA). One hundred microliters of normal swine serum (NSS) was added to the sections for 10 min to block non-specific binding of the primary antibody. 100 μ L of anti-p27^{kip1} antibody diluted 1:25 (v/v) in NSS/TBS was then applied to the test sections for 1 hr at room temperature. Positive controls consisted of multi-tissue sections containing human kidney and tonsil, with omission of the primary antibody from negative control sections. After washing with TBS, sections were incubated with 100 μ L of biotinylated goat anti-mouse/rabbit immunoglobulin (Dako Ltd, Ely, UK) diluted 1:100 in NSS for 30 min. One hundred microliters of pre-formed streptavidin-biotin/horseradish peroxidase (HRP) complex (Dako Ltd, Ely, UK) was then applied for 60 min at room temperature. Finally, bound antibody visualisation was accomplished using 3, 3'-Diaminobenzidine tetrahydrochloride (Dab, Dako Ltd, Ely, UK).

Evaluation of immunohistochemical staining

Immunohistochemical staining patterns were interpreted by two observers (DSGS and NFSW) blinded to the associated clinicopathological data. Nuclear p27^{kip1} expression was scored as follows: cases with < 10% tumor cell nuclei stained = 0, 10%-20% tumor cell nuclei stained = 1 and > 20% tumor cell nuclei stained = 2, irrespective of the staining intensity. Cytoplasmic staining was scored: complete absence of cytoplasmic staining or cytoplasmic staining of any intensity in < 10% tumor cells = 0, weak/moderate intensity cytoplasmic staining in > 10% tumor cells = 1, and intense cytoplasmic staining in > 10% tumor cells = 2. These cutoffs were based on previously published reports investigating the prognostic significance of p27^{kip1} expression in colorectal tumors^[16,25]. For the purposes of statistical analysis, these scores were re-categorized, with tumors considered either positive (score 1 or 2) or negative (score 0) for both nuclear and cytoplasmic p27^{kip1} expression. It has previously been established that in colorectal cancers, no significant differences in p27 immunoreactivity are usually seen between the centre and the invasive edge of the tumors^[26].

Statistical analysis

All calculations were performed using SPSS software (version 11.5 for Windows, SPSS Inc., Chicago, IL). Associations between categorical variables were examined using crosstabulation and the Pearson chi-square test. Kaplan-Meier curves were plotted in order to assess correlations with disease-specific survival and the significance of differences in disease-specific survival between groups was calculated using the log-rank test. Patients whose deaths related to their colorectal cancer, including early deaths from post-operative complications, were considered in the disease-specific survival calculations. Deaths resulting from non-colorectal cancer related causes were censored at the time of death. Multivariate analysis was conducted using the Cox proportional-hazards model to determine hazard ratios, and to identify variables with independent prognostic significance in this cohort. In

Table 1 Patient and tumor characteristics (*n* = 462)

Variable	Category	<i>n</i> (%)
Age (yr)	Median	72
	Range	57-89
Gender	Male	266 (58)
	Female	196 (42)
Status	Alive	169 (37)
	Dead (cancer related)	228 (49)
	Dead (unrelated causes)	64 (14)
	Unknown	1
Histological type	Adenocarcinoma	392 (85)
	Adenocarcinoma with Mucinous differentiation	51 (11)
	Adenocarcinoma with Signet ring differentiation	7 (1)
	Other	4 (1)
	Unknown	8 (2)
Histological grade	Well differentiated	29 (6)
	Moderately differentiated	353 (77)
	Poorly differentiated	71 (15)
	Unknown	9 (2)
Tumor site	Colon	238 (52)
	Rectal	181 (39)
	Unknown	43 (9)
TNM stage	0 (T _{is})	3 (1)
	1	69 (15)
	2	174 (38)
	3	155 (33)
	4	54 (12)
	Unknown	7 (2)
Extramural vascular invasion	Negative	224 (48)
	Positive	128 (28)
	Unknown	110 (24)

all cases *P* values < 0.05 were considered statistically significant.

RESULTS

Clinicopathological data

The characteristics of the 462 patients included in this study are summarized in Table 1. Male patients comprised 58% of the cohort, and the median patient age at the time of surgery was 72 years. At the time of censoring for data analysis 49% of patients had died from their colorectal cancer, with a further 14% deceased from non-colorectal cancer causes. The remaining 37% were still alive. The majority of tumors were of a moderately differentiated histological grade (77%). Similarly, 85% of tumors were adenocarcinomas, with a further 11% showing mucinous differentiation. Surviving patients had a median length follow-up of 75 (range 36-116) mo. Of the conventional clinicopathological variables, strong correlations were observed between tumor stage and disease specific survival (DSS) (Log rank = 207.33, *P* < 0.001) and between the presence of extramural vascular invasion and DSS (Log rank = 44.30, *P* < 0.001).

Of the 462 tumor specimens analyzed, 44 (9.5%) were subsequently uninterpretable due to loss of tissue from the TMA during the Immunohistochemical procedure. Among the remaining 418 tumors, three distinct patterns

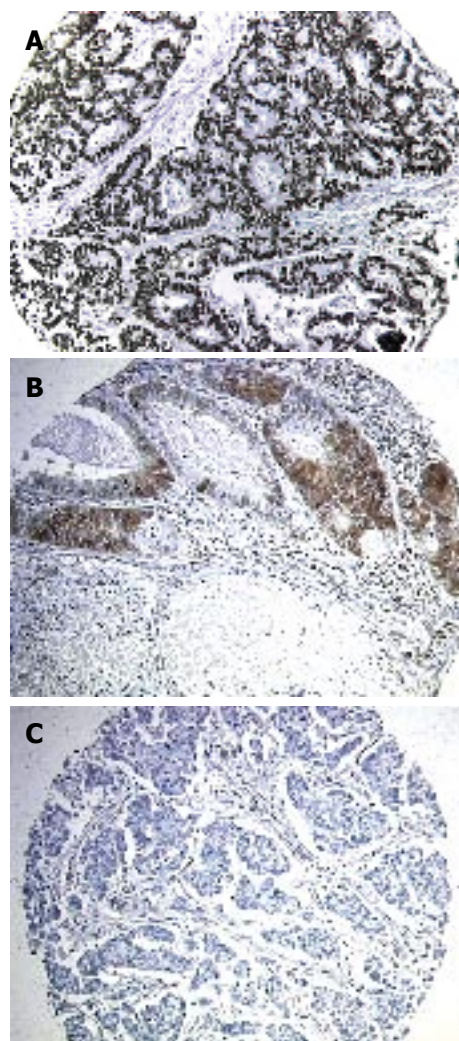


Figure 1 p27^{Kip1} expression in representative tumor samples. Tumors displayed either nuclear p27^{Kip1} alone (A), both nuclear and cytoplasmic p27^{Kip1} (B), or absent p27^{Kip1} expression (C).

of p27^{Kip1} expression were observed (Figure 1A-1C). These comprised staining of the tumor cell nuclei alone, staining of both tumor cell nuclei and cytoplasm, and absent staining of tumor elements. In contrast, no tumor cell membrane or stromal expression of p27^{Kip1} was detected in any specimen.

Nuclear p27^{Kip1} expression

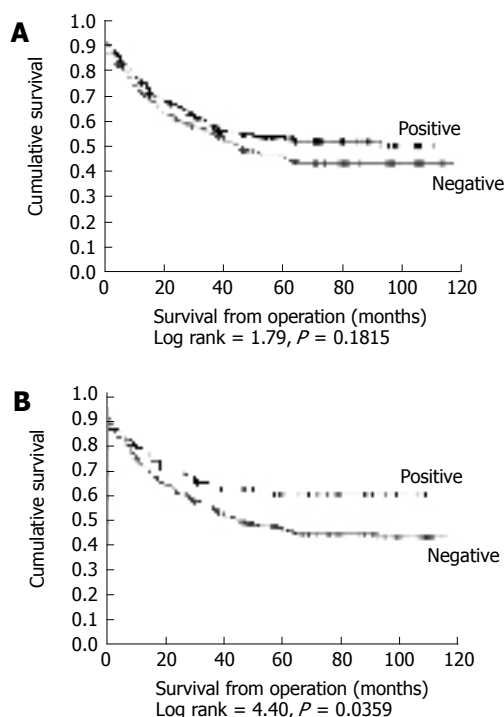
Variable expression of nuclear p27^{Kip1} was observed in 217/418 (51.9%) tumors (Table 2). In 29 of these cases nuclear p27^{Kip1} was present in 10%-20% of tumor cells within the core, and in the remainder, nuclear p27^{Kip1} was present in > 20% of tumor cells. There were no significant associations detected between any level of nuclear p27^{Kip1} expression and known clinicopathological variables, including tumor grade, stage and the presence of extramural vascular invasion. Furthermore, on Kaplan-Meier analysis, no association was found between nuclear p27^{Kip1} expression and DSS (Figure 2A, Log rank = 1.701, *P* = 0.1815).

Cytoplasmic p27^{Kip1} expression

Expression of cytoplasmic p27^{Kip1} was detected in 79/418

Table 2 p27^{kip1} localisation within 418 colorectal tumor specimens

p27 localisation	Positive <i>n</i> (%)	Negative <i>n</i> (%)	Missing <i>n</i> (%)
Nuclear	217 (51.9)	201 (48.1)	44 (9.5)
Cytoplasmic	79 (18.9)	339 (81.1)	44 (9.5)

**Figure 2** Kaplan-Meier plots for disease specific survival in relation to expression of nuclear (A) and cytoplasmic (B) p27^{kip1}.

(18.9%) tumors (Table 2). These tumors appeared to form a distinct subset of the nuclear p27^{kip1} positive tumors, as co-expression of nuclear and cytoplasmic p27^{kip1} was noted in 74/79 cases (93.6%). In 55 of the cytoplasmic p27^{kip1} positive tumors, expression was of weak or moderate intensity and in the remaining 24 cases strong expression was noted. As with nuclear p27^{kip1}, no statistically significant associations were found between the presence of cytoplasmic p27^{kip1} within the tumor and clinicopathological variables for the patient cohort. However, a significant relationship was identified between the presence of cytoplasmic p27^{kip1} and DSS. Kaplan-Meier analysis revealed that patients with cytoplasmic p27(+) tumors, had a significantly longer mean DSS of 72 (95% CI 62-83) mo, compared with a mean DSS of 62 (95% CI 57-68) mo in patients with cytoplasmic p27^{kip1}(-) tumors (Figure 2B, Log rank = 4.40, *P* = 0.0359).

Multivariate analysis

A multivariate analysis of factors influencing survival in 418 available cases was performed using the Cox proportional hazards model (Table 3). Of the conventional clinicopathological variables analysed, tumor stage (*P* < 0.001) and extramural vascular invasion status (*P* = 0.001) were demonstrated to confer independently significant

Table 3 Multivariate analysis of Cytoplasmic p27^{kip1} expression in relation to known clinical and pathological variables (*n* = 418)

Variable	Category	Hazard ratio (HR)	95% CI for HR	<i>P</i>
Gender	Female	1		
	Male	1.055	0.787-1.415	0.719
Patient age (yr)	< 65	1		
	65-79	1.262	0.843-1.889	
	80+	1.609	0.961-2.694	0.194
Tumor site	Colon	1		
	Rectal	1.117	0.813-1.535	
	Unknown	1.452	0.870-2.423	0.353
Tumor histological type	Adenocarcinoma	1		
	Non-adenocarcinoma	0.956	0.621-1.472	0.838
Tumor grade	Well differentiated	1		
	Moderately differentiated	1.167	0.582-2.337	
	Poorly differentiated	1.139	0.528-2.457	
	Unknown	0.869	0.245-3.079	0.926
Vascular invasion	Negative	1		
	Positive	1.884	1.344-2.641	
	Unknown	1.308	0.876-1.954	0.001
Tumor (TNM) stage	0/ I	1		
	II	2.021	1.102-3.708	
	III	3.741	2.066-6.774	
	IV	16.977	8.829-32.645	
	Unknown	4.211	1.049-16.902	< 0.001
Cytoplasmic p27 ^{kip1} expression	Negative	1		
	Positive	0.681	0.458-1.015	0.059

prognostic information. In this model expression of cytoplasmic p27^{kip1} within the tumor samples was associated with a strong trend towards improved DSS (HR for death in cytoplasmic p27^{kip1} positive tumors = 0.681, 95% CI 0.458-1.015), which approached statistical significance (*P* = 0.059).

DISCUSSION

p27^{kip1} is a universal CDK inhibitor that acts in G₀ and early G₁ to inhibit cyclin E/CDK2 and thereby prevents entry into the S phase of the cell cycle. It can also bind other cyclin-CDK complexes, including those involving the D-cyclins, and complexes of cyclin A/cdk2. Mitogenic growth factor signalling causes a decrease of p27^{kip1} levels and/or activity and, conversely, p27^{kip1} levels and/or activity increase in response to differentiation signals, loss of adhesion to extracellular matrix, or signalling by growth-regulatory factors such as TGF-β, c-AMP and IFN-γ. Studies in animals have shown that loss of p27^{kip1} increases the formation of tumors and also increases tumor associated deaths rates. Several studies have shown that loss of nuclear p27^{kip1} is an independent predictor of poor prognosis in colorectal cancer^[6,27,28], although other studies have failed to confirm this observation^[29,30]. Loss of nuclear

p27^{Kip1} was observed in 48% of tumors in our study. However, this showed no significant associations with known clinical or pathological variables. Additionally, on Kaplan-Meier analysis, no association was found between nuclear p27^{Kip1} expression and survival.

Several clinical studies have detected cytoplasmic expression of p27^{Kip1}, and found an inverse correlation with disease free survival^[17,28,31]. In contrast, in this large study of 418 colorectal tumors, cytoplasmic expression of p27^{Kip1} was significantly associated with a good prognosis. This may be related to the findings from recent studies showing that p27^{Kip1} can bind and inactivate Rho proteins. For tumors to metastasise, cells must alter their connections to both their neighbours and their substrate, and then migrate. Efficient migration requires a tightly balanced activation and deactivation of Cdc42, Rac and RhoA in both time and space. Indeed, two modes of tumor cell motility have been described in 3D matrices. Rho signalling through ROCK promotes a rounded bleb-associated mode of motility that does not require pericellular proteolysis. In contrast, elongated cell motility is associated with Rac-dependent F-actin-rich protrusions and does not require Rho or ROCK^[32]. Consistent with this observation are other studies that have shown inhibition of ROCK reduces the invasive behaviour of tumor cells *in vivo*^[33,34]. Sequestrations of RhoA by cytoplasmic p27^{Kip1} may inhibit RhoA, resulting in inhibition of ROCK and less aggressive tumors. In the current study, cytoplasmic expression of p27^{Kip1} was almost invariably associated with nuclear expression. These tumors may therefore have relatively controlled cell proliferation together with a reduced capacity for migration, resulting in a less aggressive tumor and a good prognosis. In contrast, loss of both cytoplasmic and nuclear p27^{Kip1} may result in uncontrolled proliferation and increased invasion, leading to an aggressive tumor and poor prognosis. Tumor cells expressing only nuclear p27^{Kip1} would have reduced proliferation but may still be invasive, resulting in an intermediate prognosis. This may explain the inconsistency of previous studies linking p27^{Kip1} expression with prognosis, as studies considering nuclear p27^{Kip1} expression alone have not identified the subgroup of tumors with additional cytoplasmic p27^{Kip1} expression. It is anticipated that the role of cytoplasmic p27^{Kip1} in promoting tumor progression may also depend upon the site and mechanism of invasion, as in a study of 61 patients with pancreatic cancer in which those with exclusively nuclear p27^{Kip1} expression were shown to have a better prognosis than those with both nuclear and cytoplasmic p27^{Kip1} expression^[31].

Recently, it has been suggested that the presence of high numbers of detached clusters of tumor cells in adjacent stroma (termed tumor budding) may serve as an adverse histopathological prognostic feature in colorectal cancer^[35]. As this tumor budding is the morphological counterpart of epithelial-mesenchymal transition, micro-invasion, and acquisition of individual cell motility, it would be of interest to correlate p27 immuno-expression patterns with colorectal tumor budding activity. However, this would be best performed using conventional whole tissue section analysis rather than TMA's. Similarly, as the size of this array does not provide us with the statistical power to co-analyze two rare events, we have not

performed analysis of DNA mismatch repair gene status and therefore cannot comment on whether cytoplasmic p27 expression may have a diverse role in colorectal tumors of differing microsatellite instability status. Finally, although out of the scope of this manuscript, it would be of interest to compare disease specific survival between p27^{Kip1} and wild-type mouse in a colorectal cancer mouse model.

The identification of novel molecular and genetic markers of prognosis will eventually allow us to provide a cancer patient with individually tailored therapy based upon the specific molecular fingerprint of his or her tumor. Our findings in a large cohort of unselected patients with colorectal cancer suggest that cytoplasmic p27^{Kip1} expression deserves further consideration as a marker of prognosis, as patients with colorectal tumors showing cytoplasmic expression of p27^{Kip1} appear to have a more favourable disease specific survival.

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Proposal of criteria to select candidates with colorectal liver metastases for hepatic resection: Comparison of our scoring system to the positive number of risk factors

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Abstract

AIM: To select accurately good candidates of hepatic resection for colorectal liver metastasis.

METHODS: Thirteen clinicopathological features, which were recognized only before or during surgery, were selected retrospectively in 81 consecutive patients in one hospital (Group I). These features were entered into a multivariate analysis to determine independent and significant variables affecting long-term prognosis after hepatectomy. Using selected variables, we created a scoring formula to classify patients with colorectal liver metastases to select good candidates for hepatic resection. The usefulness of the new scoring system was examined in a series of 92 patients from another hospital (Group II), comparing the number of selected variables.

RESULTS: Among 81 patients of Group I, multivariate analysis, i.e. Cox regression analysis, showed that multiple tumors, the largest tumor greater than 5 cm in diameter, and resectable extrahepatic metastases were significant and independent prognostic factors for poor survival after hepatectomy ($P < 0.05$). In addition, these three factors: serosa invasion, local lymph node metastases of primary cancers, and post-operative disease free interval less than 1 year including synchronous hepatic metastasis, were not significant, however, they were selected by a stepwise method of Cox regression analysis ($0.05 < P < 0.20$). Using these six variables, we created a new scoring formula to classify patients with colorectal liver metastases. Finally, our new scoring system not only classified patients in Group I very well, but also that in Group II, according to long-term outcomes after hepatic resection. The positive number of these six variables also classified them well.

CONCLUSION: Both, our new scoring system and the positive number of significant prognostic factors are useful to classify patients with colorectal liver metastases in the preoperative selection of good candidates for hepatic resection.

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Key words: Colorectal cancer; Liver metastasis; Hepatic resection; Prognostic factor

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INTRODUCTION

It is well accepted that hepatic resection for colorectal liver metastases is beneficial; it has been reported survival rates improve to as high as almost 30% to 50% at 5 years after curative hepatic resection recently^[1-10]. However, the feasible criteria for surgery are still controversial. The purpose of the present study is to implement a useful classification system to select optimal candidates for hepatic resection among patients with colorectal liver metastases.

MATERIALS AND METHODS

Patients

Group I : Between January 1, 1981 and March 31, 1997, 83 consecutive patients underwent partial hepatectomy for colorectal liver metastases at the First Department of Surgery, presently the Department of Surgical Oncology, Tokyo University Hospital. Every hepatectomy was considered curative because of complete macroscopic resection of hepatic tumors. Even though extrahepatic metastases were excised, hepatectomy was performed if extrahepatic metastatic tumors could be completely resected as well macroscopically. During the post-operative period, two patients died in the hospital. One died secondary to aspiration pneumonia and the other by

developing severe intra-abdominal sepsis (mortality rate: 3.1%). Therefore, the remaining 81 patients were followed until death or their last outpatient visit up to December 31, 2004. The follow-up period ranged from 4 to 197 mo. with a median of 53.0 mo. After discharge from the hospital, patients were closely monitored at the outpatient clinic or affiliated institutions. During visits, measurement of serum carcinoembryonic antigen (CEA) levels and ultrasonography were performed at least once every two months for early detection of recurrence. In addition, computed tomography was performed approximately twice a year. Almost all cases of cancer recurrence were diagnosed by these investigative tests. When the diagnosis was unclear, angiography and/or needle biopsy under ultrasonic guidance was performed to establish a firm diagnosis.

Group II: Between January 1, 1981 and December 31, 2003, 92 consecutive patients underwent partial hepatectomy for colorectal liver metastases at the Department of Surgery, Teikyo University Hospital. During the post-operative period, two patients died in the hospital from post-operative hepatic failure (mortality rate: 5.6%). The remaining 92 patients were followed up until death or their last outpatient visit up to December 31, 2004. The follow-up period ranged from 4 to 110 mo. with a median of 39.0 mo. After discharge from the hospital, each patient was closely monitored at the outpatient clinic or affiliated institutions in a manner similar to the patients at University of Tokyo hospital.

Prognostic factors

We examined the statistical significance of thirteen factors that were expected to influence long-term prognosis in patients in Group I. Each factor could only be determined preoperatively or during surgery. These included gender; male or female, age at hepatectomy; < 60 or ≥ 60 years, chronology of hepatic metastases; synchronous or metachronous, and post-operative disease free interval, ≤ 1 year including synchronous metastasis or > 1 year; number of hepatic metastases; solitary or multiple, maximum diameter of the hepatic metastases; ≤ 5 cm or > 5 cm, unilobar or bilobar hepatic involvement, resectable extrahepatic distant metastasis, which included pulmonary metastases, localized peritoneal metastases, or hepatoduodenal lymph node metastases, serum carcinoembryonic antigen level (CEA) at hepatectomy; less or higher than 10 times of the upper normal level, serosa invasion, and regional lymph node metastases of the primary colorectal carcinoma, type of hepatic resection; lobectomy or limited resection less than lobectomy, and macroscopical surgical resection margin; < 1 cm or ≥ 1 cm. The demographic characteristics and tumor-related features, which are statistically analyzed later, are summarized in Table 1.

Statistical analysis

Survival rates after hepatectomy were calculated using data obtained from patients in Group I and Group II by the Kaplan-Meier method. Only deaths attributable to recurrent cancer were treated as events. Patients who died secondary to other causes without recurrence were treated

Table 1 Prognostic factors entered into multivariate analysis based on proposed clinical and histopathological features

Variable	n
Gender (male/female)	61/20
Age at hepatectomy($< 60/\geq 60$ yr)	32/49
Chronology of hepatic metastasis (synchronous/metachronous)	41/40
Disease free interval after colectomy (synchronous or ≤ 1 yr/ > 1 yr)	56/25
Extrahepatic distant metastases (no/yes)	9/72
CEA (≤ 10 times of normal value/ > 10 times of normal value)	57/24
Primary lesion	
Depth of invasion (up to subserosa/more)	57/24
Lymph node metastasis (no/yes)	39/42
Hepatic metastasis	
Number (single / multiple)	45/36
Maximum diameter (≤ 5 cm/ > 5 cm)	59/22
Lobe involved (unilobar/bilobar)	64/17
Therapeutic factor	
Type of hepatectomy (limited/lobectomy)	56/25
Surgical margin (< 1 cm / ≥ 1 cm)	61/20

as censored. Selection of independent and significant prognostic variables was performed by multivariate analysis in patients of Group I with the stepwise analysis of Cox proportional hazard regression model. The stepwise variable selection was performed at a 0.20 significant level in this study. The creation of a scoring formula was based on the results of the multivariate analysis as above. Each selected independent and significant prognostic factor was respectively given a coefficient. The total score of each patient ($y1$) was calculated according to a formula that consisted of these selected prognostic factors.

The scoring formula was applied to patients in Group II as well as Group I. Patients were divided into sub-groups depending on their total scores ($y1$) as follows; $y1 = 0, 0 < y1 \leq 1, 1 < y1 \leq 2, 2 < y1 \leq 3, 3 < y1 \leq 4, 4 < y1 \leq 5, 5 < y1 \leq 6, 6 < y1$. Survival rates according to the sub-groups were calculated using the Kaplan-Meier method. On the other hand, survival rates according to the positive number of selected significant prognostic factors ($y2$) were also calculated, and compared to the results of the scoring formula ($y1$). They were analyzed using the log-rank test.

RESULTS

The overall cancer-related survival rates after surgical resection in the 81 patients in Group I were 88.2% at 1 year, 67.7% at 2 years, 56.0% at 3 years, 51.6% at 4 years, and 49.1% at 5 years, respectively (Figure 1). The results from the multivariate analysis of those variables expected to influence cancer-related survival after surgical resection in Group I are provided in Table 2. Only variables selected by the stepwise analysis of the Cox proportional hazard regression model are shown in the table ($P < 0.20$). Multiple hepatic metastatic tumors, hepatic metastatic tumors that were greater than 5 cm in maximum diameter, and resectable extrahepatic distant metastases were significant and independent variables that influenced cancer-related survival ($P < 0.05$). In addition, serosa

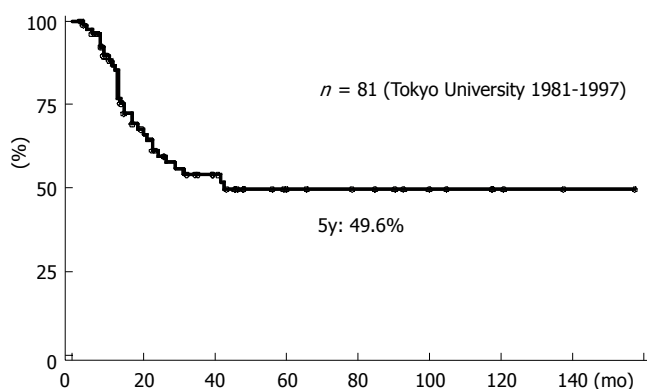


Figure 1 Kaplan-Meier cancer-related survival curve after hepatic resection for colorectal liver metastases in patients of Group I.

Table 2 Multivariate analysis for clinicopathological variables with the stepwise analysis of Cox proportional hazard regression model $n = 81$ (Tokyo Univ 1981-1997)

Variable	Parameter	P	Hazard ratio (95% CI)
Diameter > 5 cm	1.35657	0.0013	3.883 (1.703-8.852)
Extrahep met (+)	1.19430	0.0133	3.301 (1.282-8.502)
Number ≥ 2	0.85412	0.0265	2.349 (1.105-4.997)
DF interval < 1 yr	0.67838	0.1602	1.971 (0.765-5.080)
n (+) of primary	0.60034	0.1352	1.823 (0.829-4.007)
\geq se (+) of primary	0.54877	0.1676	1.731 (0.794-3.774)

$P > 0.2$; Age ≥ 60 , Gender, Synchronous, Bilobar invasion, CEA, Major hepatectomy (lobectomy), Surgical margin < 1 cm.

Table 3 The scoring formula (y_1) and the positive number of risk factors (y_2)

Score (y_1) = $1.35657 \times (\text{Diameter} > 5 \text{ cm})$
 $+ 1.19430 \times (\text{Extrahep.met})$
 $+ 0.85412 \times (\text{Multiple})$
 $+ 0.67838 \times (\text{D.F.I.} < 1 \text{ yr})$
 $+ 0.60034 \times (n+ \text{ of Primary})$
 $+ 0.54877 \times (\geq \text{se of Primary})$

$y_1: 0 \leq y_1 \leq 5.2324$

Score (y_2) = Number of Factors

$y_2: 0, 1, 2, 3, 4, 5, 6$

invasion and regional lymph nodes metastases of primary colorectal cancer, and recurrent hepatic metastases within one year after resection of primary colorectal cancer including synchronous hepatic metastases were considered close to significant ($0.05 < P < 0.20$).

As described above, multivariate analysis indicated six variables that more independently influenced cancer-related survival after hepatic resection. In the next step of creating a scoring formula, we use these six variables to classify patients as shown in Table 3. In this formula, each variable had a coefficient, which was indicated as a parameter estimate by Cox regression analysis, as shown in Table 2. If the factor was positive, it was given a score of one point. If it was negative, it was given a score of 0 points. Thereafter the total score of each patient (y_1) could be calculated. On the other hand, the positive number of

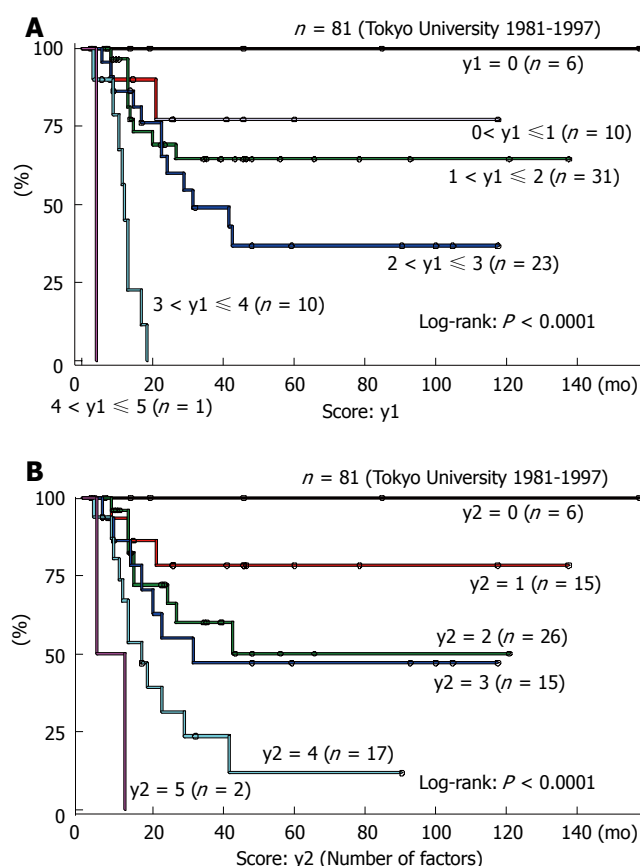


Figure 2 Kaplan-Meier cancer-related survival curves after hepatic resection for colorectal liver metastases. **A:** According to the total score (y_1) in patients of Group I; **B:** According to the positive number of risk factors (y_2) in patients of Group I.

these six variables (y_2) of each patient was also calculated

Classification of patients and survival

The total scores of patients (y_1) in Group I were distributed between 0 and 5.2324. The survival curves of patients based on the total score (y_1) are shown in Figure 2A. Which were accurately and significantly classified ($P < 0.0001$). We clearly found that the prognosis of patients with a total score of (y_1) > 3 were very poor. On the other hand, the survival curves of patients based on the positive number (y_2) are shown in Figure 2B. They were well and significantly classified as well ($P < 0.0001$). We clearly found that the prognosis of patients with positive number ≥ 5 were very poor.

The survival curves of all patients in Group II are shown in Figure 3. The overall cancer-related survival rates of the 92 patients in Group II after surgical resection were 83.2% at 1 year, 62.6% at 2 years, 49.4% at 3 years, 41.0% at 4 years, and 36.4% at 5 years, respectively. We applied our scoring system to 92 patients in Group II. The total scores of these patients (y_1) were distributed between 0 and 5.2324. The survival curves of patients based on the total score (y_1) are shown in Figure 4A. They were also significantly classified ($P < 0.0001$). We clearly found that the prognosis of patients with total score (y_1) > 3 were very poor. On the other hand, the survival curves of patients based on the positive number (y_2) are shown in Figure 4B. They were significantly classified as well ($P < 0.0001$). We

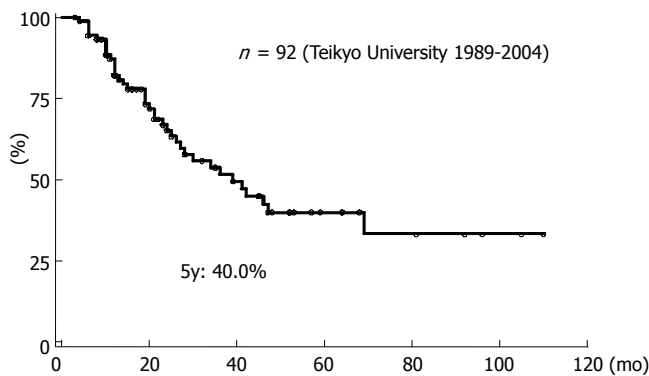


Figure 3 Kaplan-Meier cancer-related survival curve after hepatic resection for colorectal liver metastases in patients of Group II.

clearly found that the prognosis of patients with positive number ≥ 5 were very poor.

DISCUSSION

Clinical and pathological factors have been identified as important prognostic determinants of survival after surgical resection for colorectal liver metastases. These include sex^[11], age at hepatectomy^[1,12], stage of the primary tumor^[1,3,4,11-15] (including local lymph node metastases and depth of invasion), synchronous or metachronous hepatic metastases^[1-3,6,8,10,12,13,16] (including disease free interval between resection primary tumor and presence of hepatic metastasis), number^[1-4,6,7,11,12,15,17-19], size^[1,2,8,9,11,13,17,20], and distribution^[6,12,14] of hepatic metastases; serum carcinoembryonic antigen (CEA) level^[1,3,4,7,20,21], extrahepatic distant metastases^[4-6,8,10,11,15,20] (including lymph node metastasis of hepatic hilum), type of hepatectomy^[14], surgical margin^[5,7,9,11-13,15,17,18,20], and adjuvant chemotherapy^[8,22,23].

In the present study, we aimed to create a new classification system of patients with colorectal liver metastases to distinguish patients who would be good candidates for hepatic resection. There have been some classification systems for these patients so far^[1-3,12], however, they are not accepted world wide yet. We selected clinicopathological features that could be recognized as prognostic factors only before or during surgery, because we had to decide how to treat those patients pre- or intraoperatively. We included some therapeutic factors as well in this study because we intended to determine whether those therapeutic factors were significant or not. We believed that any therapeutic factors that may be significant should also be included in the classification scoring system. However, we excluded adjuvant chemotherapy because it has been arbitrarily performed in past years and we could not evaluate its efficacy. We considered positive surgical margins to be a very important factor^[5], however, we excluded it because it was an incidental factor and could only be recognized after surgery.

Among the thirteen clinicopathological factors we proposed, we found and selected the following to be more significant and independent prognostic factors for poor survival after hepatectomy: multiple hepatic metastatic tumors; hepatic metastatic tumor greater than 5 cm in

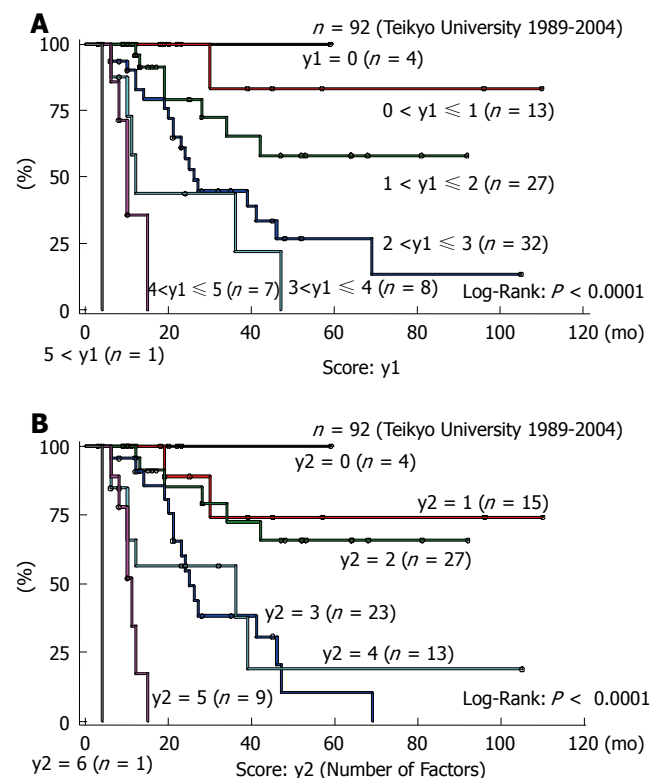


Figure 4 Kaplan-Meier cancer-related survival curves after hepatic resection for colorectal liver metastases. **A:** According to the total score ($y1$) in patients of Group II. **B:** According to the positive number of risk factors ($y2$) in patients of Group II.

diameter; resectable extrahepatic distant metastases; recurrent hepatic metastases within one year after resection of primary colorectal cancer including synchronous hepatic metastases; regional lymph node metastases and serosa invasion of primary colorectal cancers. On the other hand, none of the therapeutic factors we proposed were selected as significant prognostic factors. We found that a 1 cm negative surgical margin and type of hepatectomy were not important as long as the tumor was resected completely for colorectal liver metastases. The goal of the present study was to establish a new scoring system to classify patients with colorectal liver metastases. Hence, this would allow an accurate prediction of long-term prognosis after surgical resection in these patients. Such a system would enable better selection of candidates for surgical resection. At last, our new scoring system, which includes six prognostic factors described above, significantly classified not only a group of patients in one hospital but also a group of patients in another hospital. We are not aware of a similar scale system for predicting the long-term outcome of surgical resection for colorectal liver metastases. According to our scoring system, patients with a total score of $0 \leq y1 \leq 3$ were suitable candidates for hepatectomy. On the other hand, patients with a total score of $y1 > 5$ should not undergo hepatectomy. We believe that patients with a total score of $3 < y1 \leq 5$ should undergo hepatectomy first, since there is no other treatment superior to hepatectomy at present.

In addition, we realized that the positive number of significant prognostic factors also classified patients well with colorectal liver metastases. The prognosis of pa-

tients with positive number ≥ 5 was very poor. It seemed that our scoring formula and the positive number of risk factors had almost equal usefulness to select good candidates for hepatic resection at present. The former is more accurate, but complicated to calculate, while the latter is less accurate, but simple. Further investigation should be necessary to prove their superiority.

In conclusion, we identified six important and independent prognostic factors that were recognized before or during surgery. We used multivariate analyses from a retrospective review of patients who underwent hepatic resection for colorectal liver metastases in one hospital. We proposed a new scoring system to classify those patients according to the long-term outcome after hepatectomy. Our scoring system accurately classified a different group of patients from another hospital as well. In addition, the positive number of significant prognostic factors also classified with those patients with good accuracy. Further investigation is necessary in the future.

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H pylori

Does delayed gastric emptying shorten the *H pylori* eradication period? A double blind clinical trial

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RESULTS: Thirty patients were excluded. Per-protocol analysis showed successful eradication in 53% in group 1, 56% in group 2, 58% in group 3, 33.3% in group 4, 28% in group 5, and 53% in group 6. Eradication rate, patient compliance and satisfaction were not significantly different between the groups.

CONCLUSION: It seems that adding sugar or levodopa or both to anti *H pylori* eradication regimens may lead to shorter duration of treatment.

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Key words: *H pylori*; Gastric emptying; Glucose; Levodopa

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Abstract

AIM: To evaluate the gastric emptying inhibitory effects of sugar and levodopa on *H pylori* eradication period.

METHODS: A total of 139 consecutive patients were randomized into 6 groups. The participants with peptic ulcer disease or non-ulcer dyspepsia non-responding to other medications who were also *H pylori*-positive patients either with positive rapid urease test (RUT) or positive histology were included. All groups were pretreated with omeprazole for 2 d and then treated with quadruple therapy regimen (omeprazole, bismuth, tetracycline and metronidazole); all drugs were given twice daily. Groups 1 and 2 were treated for 3 d, groups 3, 4 and 5 for 7 d, and group 6 for 14 d. Groups 1 to 4 received sugar in the form of 10% sucrose syrup. Levodopa was prescribed for groups 1 and 3. Patients in groups 2 and 4 were given placebo for levodopa and groups 5 and 6 received placebos for both sugar and levodopa. Upper endoscopy and biopsies were carried out before treatment and two months after treatment. Eradication of *H pylori* was assessed by RUT and histology 8 wk later.

INTRODUCTION

H pylori has been implicated as a predisposing factor in gastric cancer, chronic active gastritis, duodenal ulcer, gastric ulcer and gastric lymphoma^[1]. The incidence rate of stomach cancer in Iran is high, well above the world average; it is the most common cancer in males and the third one in females^[2]. Also, the reinfection rate in our country is high enough (20%, 3 years after successful eradication)^[1] to justify wide investigations to find safe and short *Helicobacter* treatments. However, *H pylori* eradication is a multifactor problem and depends on histological findings and ulcer depth, kind of medication and duration of treatment, age, patient compliance, genetic predisposition, geographical area of living, *H pylori* resistance, non-steroid anti-inflammatory drugs (NSAIDs) exposure and finally stomach dynamicity (gastric emptying)^[3-16].

The current approach to the patient with suspected *H pylori* infection consists of an adequate indication to test for the presence of the infection, choice of an appropriate antimicrobial regimen, and education about its use and

side effects, followed by post-therapy testing to confirm cure^[17]. In order to achieve the best results from this approach, patient compliance is of outmost importance and the favored regimen should have the least side effects; otherwise, treatment failure may ensue^[3,18].

H pylori infection plays a role in gastric emptying in type 1 diabetic patients; a delay in gastric emptying is observed with the disappearance of gastritis associated with *H pylori* infection after eradication treatment in those patients^[19]. In contrary, others have shown that *H pylori* infection is not associated with delayed gastric emptying in diabetes^[20,21]. Barnett *et al*^[22] revealed that not only gastroparesis does not predispose to *H pylori* infection or chronic gastritis, but also there is a significantly lower incidence of *H pylori* in individuals with delayed gastric emptying compared to those with normal emptying (5% *vs* 31%, respectively). Moreover, there is no link between anxiety and gastric emptying in chronic duodenal ulcer whether in “fast” emptiers ($t_{1/2}$ less than 90 min) or “normal” emptiers ($t_{1/2}$ more than 90 min)^[23]. Thus, inhibition of gastric emptying by physiologic and pharmacological methods may enhance local delivery of therapeutic agents to the stomach which is a useful strategy in the treatment of *H pylori* infection^[24-27] especially given that *H pylori* is found both attached to mucous cells of the human stomach and under the mucous layer; there is no evidence that attachment of *H pylori* to eukaryotic cells increases their resistance to antibiotics compared with free-floating bacteria^[28] and that omeprazole may displace *H pylori* from the antrum to the stomach body which could interfere with colonization studies in patients receiving the drug^[17]. However, omeprazole has shown no significant effect on solid or liquid gastric emptying in duodenal ulcer^[29]. Also, various alterations of gastric emptying in duodenal ulcer have been demonstrated by different studies^[30-33].

Sugar^[34-39] and levodopa^[40] are among the safest agents that prolong gastric emptying. Glucose in the pylorus has an inhibitory effect on gastric emptying which will be even slower with progressive increases in glucose concentration^[36,37]. Dopamine receptor antagonists (metoclopramide, domperidone) also play an important role in the treatment of gastric emptying disorders^[41].

One of the problems of the currently used anti-*H pylori* drug regimens is their long period of treatment which will be accompanied by more frequent side effects and lower compliance. In this study, we aimed to evaluate the gastric emptying inhibitory effects of sugar and levodopa on *H pylori* eradication period.

MATERIALS AND METHODS

Design

This is a double blind randomized placebo-controlled mono-center trial with 6 parallel groups which was conducted at a university hospital.

Participants

Patients with peptic ulcer disease or non-ulcer dyspepsia non-responding to other medications who were also *H pylori*-positive either with positive rapid urease test (RUT)

or positive histology were included in the study. Written informed consent was obtained from all patients. Since administration of glucose in diabetic patients is not ethical and both gastroparesis and accelerated gastric emptying are seen in these patients^[20,42], those with diabetes mellitus or abnormal fasting blood sugar were not included in the study. The same was applied to those who received NSAIDs or those with other systemic diseases in which quadruple therapy was harmful, such as epilepsy. Exclusion criteria were refusal to undergo re-endoscopy, medication side effects and non-compliance.

Interventions

A total of 139 consecutive patients, who were eligible to undergo *H pylori* eradication based on endoscopic findings, were randomized into 6 groups (Table 1). The subjects' gastrointestinal signs and symptoms were recorded. They were then referred to the clinical pharmacist responsible for prescriptions and completion of the questionnaires on medication side effects and compliance. All groups were pretreated with omeprazole (20 mg bid administered at 6 AM and 6 PM) for 2 d and then, treated with quadruple therapy; i.e. omeprazole (20 mg bid administered at 6 AM and 6 PM), bismuth (240 mg bid administered at 6:35 AM and 6:35 PM), tetracycline (750 mg bid administered at 6:35 AM and 6:35 PM) and metronidazole (500 mg bid administered at 6:35 AM and 6:35 PM); groups 1 and 2 were treated for 3 d, groups 3, 4 and 5 for 7 d, and group 6 for 14 d. Groups 1 to 4 received sugar in the form of 10% sucrose syrup. Since glucose solutions empty biphasically, rapidly for the first minutes, then slowly and proportionately to glucose concentration to deliver glucose calories through the pylorus at a regulated rate (0.4 kcal/min)^[38], we administered 125 cc of solution, 5 min before taking medications (6:30 AM and 6:30 PM) and then, 375 cc with medications. For groups 1 and 3 sinemet (levodopa 125 mg plus 62.5 mg carbidopa) was administered twice daily at 6 AM and 6 PM. Patients in groups 2 and 4 were given placebo for levodopa and groups 5 and 6 received two placebos for sugar and levodopa. All patients were repositioned to prone, supine, left and right lateral decubitus positions and remained in each position for 5 min twice daily at 7 AM and 7 PM. Each of the *H pylori* eradication regimens was followed by famotidine administration for 4 wk (40 mg/d at bedtime).

Outcome measures

Upper endoscopy was carried out before treatment and two months after initiation of treatment. This was to allow for a 6-wk antibiotic-free period and also a 2-wk antisecretory-free period before re-evaluating *H pylori* positivity. Four antral (two for RUT and two for histology) and four corpus (two for RUT and two for histology) biopsy specimens were taken. Eradication of *H pylori* was considered as the main outcome measure which was assessed by RUT and histology 8 wk after initiation of intervention^[43]. Also, peptic ulcer symptoms and signs as well as endoscopic findings were recorded by gastroenterologist before, and 8 wk after initiation of treatment. Medication side effects were asked and recorded before starting and after ending

Table 1 Summary of drug regimens administered double-blindly for 6 groups

Group	Quadruple therapy	Pretreatment with omeprazole for 2 d	Sugar (10% sucrose)	Levodopa (Sinemet)	Placebo for sugar	Placebo for levodopa	Duration of treatment
1	✓	✓	✓	✓			3
2	✓	✓	✓			✓	3
3	✓	✓	✓	✓			7
4	✓	✓	✓			✓	7
5	✓	✓			✓	✓	7
6	✓	✓			✓	✓	14

Table 2 Comparison of the frequencies of peptic ulcer symptoms before and two months after initiation of intervention

	Pyrosis		Abdominal pain		Night awakening pain		Flatulence		Early hungeriness		Early satiety		Fullness		Severe weight loss	
	Before therapy	After therapy	Before therapy	After therapy	Before therapy	After therapy	Before therapy	After therapy	Before therapy	After therapy	Before therapy	After therapy	Before therapy	After therapy	Before therapy	After therapy
Group 1 (SL3)	12	0	16	0	11	0	11	4	13	1	6	1	11	2	4	0
Group 2 (SP3)	9	2	16	2	12	0	10	0	11	2	5	0	12	2	1	0
Group 3 (SL7)	11	3	16	3	15	0	10	1	12	2	7	0	15	4	5	2
Group 4 (SP7)	10	4	16	5	8	1	12	3	7	0	8	0	13	5	4	0
Group 5 (PP7)	14	2	12	2	9	0	13	2	8	1	7	0	14	3	4	0
Group 6 (PP14)	7	3	16	4	8	1	10	2	11	2	4	1	11	3	3	2

Group 1: Quadruple Therapy + Sugar (S) + Levodopa (L) for 3 d; Group 2: Quadruple Therapy + Sugar + Placebo (P) for 3 d; Group 3: Quadruple Therapy + Sugar + Levodopa for 7 d; Group 4: Quadruple Therapy + Sugar + Placebo for 7 d; Group 5: Quadruple Therapy + Placebo + Placebo for 7 d; Group 6: Quadruple Therapy + Placebo + Placebo for 14 d.

the period of each regimen by the clinical pharmacist who was also responsible for inquiring about patient compliance.

Statistical analysis

Kruskal-Wallis test was used to assess differences in eradication rates, rates of medication side effects, frequencies of symptoms and endoscopic signs, patient compliance and satisfaction rates among 6 groups. Wilcoxon test was applied to evaluate the above-mentioned factors within the groups before and after intervention. ANOVA and paired *t*-test were used to find the differences in size and number of ulcers between, and within the groups, respectively. *P* value of less than 0.05 was taken as significant.

RESULTS

Thirty patients were excluded from the study either due to refusal to undergo re-endoscopy (18 patients) or because of medication side effects (11 patients) or non-compliance (interruption of therapy, 1 patient).

The frequencies of symptoms before and after intervention were not significantly different between the groups but were all significant within the groups except for severe weight loss (Table 2). The only side effect showing

significant difference between the groups was nausea (*P* < 0.05), which was least frequent in groups 2 and 1 (56% and 59% respectively) and most frequent in group 6 (79%), 5 (73%) and 3 (70%). This comparison shows that the duration of treatment has a direct relationship with nausea. Other reported side effects included vomiting, anorexia, dry mouth, metal taste, diarrhea, tinnitus, headache, dyskinesia, vertigo, paresthesia and insomnia.

The frequencies of endoscopic results including fundal erythema and ulcer, antral erythema and ulcer, body erythema and ulcer, duodenal erythema and ulcer, ulcer hemorrhage and number and size of ulcers before and after intervention did not show any significant difference between the groups. On the other hand, treatment was significantly effective in all groups because eradication rates, ulcer numbers and ulcer sizes were significantly different within the groups. Index of successful *H. pylori* eradication was defined as negative RUT plus negative pathology report; both were evaluated 8 wk after the first day of intervention. Per-protocol analysis showed successful eradication in 10/19 (53%) patients in group 1, 10/18 (56%) in group 2, 11/19 (58%) in group 3, 6/18 (33.3%) in group 4, 5/18 (28%) in group 5, and 9/17 (53%) in group 6. Intention-to-treat analysis resulted in successful eradication in 10/20 (50%) patients in group 1, 10/20 (50%) in group 2, 11/21 (52.5%) in group 3, 6/20 (30%) in

group 4, 5/20 (25%) in group 5, and 9/20 (45%) in group 6. Eradication rate, patient compliance and satisfaction rates were not significantly different between the groups.

DISCUSSION

Multiple therapeutic regimens involving different combinations of omeprazole, lansoprazole, ranitidine, famotidine, amoxicillin, bismuth, clarithromycin, furazolidone and metronidazole have been tested during various periods of time to determine the optimal regimen(s) for *H pylori* eradication, leading to very different results depending on the geographical area.

A group of gastroenterologists at Jichi Medical School, Japan, instilled triple antibiotics (bismuth subnitrate, amoxicillin and metronidazole) plus pronase into the stomach through a nasally introduced intestinal tube. They showed that 1 to 2 h of topical therapy was a safe, effective, and well-tolerated procedure for the treatment of *H pylori* infection^[25-27]. In another study, by contrast, the 1-h topical method with the same antibiotics and pronase did show low eradication rates in patients with duodenal ulcer. In addition, the topical treatment was characterized by a high rate of side effects and poor tolerance^[44]. On the other hand, Atherton *et al*^[24] demonstrated that dosing after food profoundly prolongs gastric residence of the drug label and also improves intragastric distribution by increasing delivery to the body and fundus. Omeprazole enhanced the effect of food, but had no effect in fasted subjects. Post-prandial dosing may, therefore, be useful for improving delivery of some anti-*H pylori* agents.

Currently, successful cure of *H pylori* infection requires 2 or more antibiotics and the ideal duration of therapy is unknown, but in general, some believe that therapy for one-week or less is not as effective as therapies of longer durations^[3,17,45-51]. Meanwhile, others have shown one-week treatment or even shorter could be curative^[52-65]. Our findings revealed that both 7 d and 14 d of metronidazole-based quadruple therapy with no sugar or levodopa resulted in suboptimal (< 75%)^[66] eradication of *H pylori* infection which can be explained by the high rate of metronidazole resistance in Iran (50%-60%)^[66-68]. Clarithromycin is expensive and has low availability in Iran^[67,69] and there is a nearly 20%-25% rate of resistance to this macrolide^[68,70] despite the fact that clarithromycin has not yet been introduced to the Iranian formulary as a generic drug. Furazolidone may also interfere with levodopa^[71]. In countries with a high prevalence of metronidazole resistance, higher doses of metronidazole^[72] and increased drug concentration in situ^[73] augment the eradication rate of *H pylori*. All of these factors and the 0% dual resistance of *H pylori* to metronidazole and tetracycline^[68] led us to use metronidazole-based quadruple therapy combined with delayed gastric emptying in the current study. We found only one study in the literature using levodopa to delay gastric emptying in the presence of *H pylori* which showed pretreatment with levodopa would not modify either the sensitivity or the specificity of the urease breath test in identifying *H pylori* infection^[74].

In conclusion, shorter duration of *H pylori* eradication in groups 1, 2 and 3 (which included sugar, levodopa or

both) combined with the eradication rate seen with the 14-d regimen (with no sugar or levodopa) indicates that adding sugar or levodopa to anti-*H pylori* regimens through increasing gastric emptying time may lead to short-term treatments. Nonetheless, there were less than 30 subjects in each group which is the minimum requirement for normal distribution. Further evaluation with larger sample size is warranted.

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BASIC RESEARCH

Effect of transforming growth factor- β 1 on human intrahepatic cholangiocarcinoma cell growth

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TGF- β 1 activates IL-6 production, and the functional interaction between TGF- β 1 and IL-6 contributes to ICC cell growth by TGF- β 1.

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Key words: Transforming growth factor- β 1; Interleukin-6; Intrahepatic cholangiocarcinoma

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Abstract

AIM: To elucidate the biological effects of transforming growth factor- β 1 (TGF- β 1) on intrahepatic cholangiocarcinoma (ICC).

METHODS: We investigated the effects of TGF- β 1 on human ICC cell lines (HuCCT1, MEC, and HuH-28) by monitoring the influence of TGF- β 1 on tumor growth and interleukin-6 (IL-6) expression in ICC cells.

RESULTS: All three human ICC cell lines produced TGF- β 1 and demonstrated accelerated growth in the presence of TGF- β 1 with no apoptotic effect. Studies on HuCCT1 revealed a TGF- β 1-induced stimulation of the expression of TGF- β 1, as well as a decrease in TGF- β 1 mRNA expression induced by neutralizing anti-TGF- β 1 antibody. These results indicate that TGF- β 1 stimulates the production and function of TGF- β 1 in an autocrine fashion. Further, IL-6 secretion was observed in all three cell lines and exhibited an inhibitory response to neutralizing anti-TGF- β 1 antibody. Experiments using HuCCT1 revealed a TGF- β 1-induced acceleration of IL-6 protein expression and mRNA levels. These findings demonstrate a functional interaction between TGF- β 1 and IL-6. All three cell lines proliferated in the presence of IL-6. In contrast, TGF- β 1 induced no growth effect in HuCCT1 in the presence of small interfering RNA against a specific cell surface receptor of IL-6 and signal transducer and activator of transcription-3.

CONCLUSION: ICC cells produce TGF- β 1 and confer a TGF- β 1-induced growth effect in an autocrine fashion.

INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is the second most common form of primary liver cancer after hepatocellular carcinoma. Despite advances in diagnosis and treatment, the prognosis of ICC has not yet been resolved^[1-3]. When compared with other malignancies, ICC is generally characterized by strong proliferation, invasion, and early metastasis. To improve the prognosis, we require a fuller understanding of the molecular mechanisms behind its proliferation and progression.

Transforming growth factor- β 1 (TGF- β 1) is a multifunctional polypeptide with potent effects as a growth inhibitor for most epithelial cells^[4]. TGF- β 1 has been shown to induce cell arrest and fibrosis in hepatocytes^[5-8]. While TGF- β 1 expression is normally low in normal intrahepatic biliary cells, it has been found to markedly increase in inflammatory and obstructive lesions of the bile duct^[9,10]. There are also reports suggesting that the TGF- β 1 signaling system plays a role in carcinogenesis and cancer progression^[11,12]. Patients with breast cancer^[13,14] and hepatocellular carcinoma^[15] have shown elevated concentrations of TGF- β 1 in serum. TGF- β 1 and its receptor mRNA were confirmed to be expressed at elevated levels in an animal model of ICC^[15,16], and the former has also been found to be elevated in human ICC^[17,18]. These data seem to conflict with the well-known mitoinhibitory effect of TGF- β 1, and the mechanism and function of TGF- β 1 in carcinogenesis remain poorly

understood. In this study we investigated the biological effects of TGF- β 1 on ICC by monitoring the influences of TGF- β 1 on tumor growth and interleukin-6 (IL-6) expression in human ICC cells.

MATERIALS AND METHODS

Cells and culture conditions

Three human intrahepatic cholangiocarcinoma cell lines, HuCCT1, MEC, and HuH-28, and a mink lung epithelial cell line, Mv1Lu, were kindly provided by the Cell Resource Center for Biomedical Research, Tohoku University, and used for the various experiments in this study. HuCCT1 and MEC were cultured in RPMI1640 (Sigma-Aldrich Co., St. Louis, MO), HuH-28 was cultured in MEM (GIBCO, Rockville, MD), and Mv1Lu was cultured in EMEM-NEAA (GIBCO). All medium was supplemented with 100 units/mL penicillin, 100 μ g/mL streptomycin (GIBCO), and 10% fetal calf serum (PAA Laboratories GmbH, Pasching, Austria). Cells were grown as subconfluent mono-layers in a humidified atmosphere containing 5% CO₂ at 37°C and passaged using a treatment with 0.25% trypsin every 7 d.

Immunofluorescence study

HuCCT1 cells grown to 70% confluency on slide glass were fixed in Zamboni solution at room temperature for 30 min. After a specific antibody against TGF- β 1 (rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), TGF- β receptor II (rabbit polyclonal, Santa Cruz Biotechnology), and IL-6 (mouse monoclonal, Genzyme, Cambridge, MA) were added for 45 min at 37°C, Alexa Fluor 488-conjugated anti-rabbit antibody (Invitrogen Corp., Carlsbad, CA) was used as a second antibody for 30 min at 37°C. Finally, cells were stained for 30 min at room temperature with 4, 6-diamidino-2-phenylindole (DAPI). Pictures were taken using a cooled CCD camera and MetaMorph Imaging Software (Universal Imaging Corporation, Downingtown, PA) with equal exposure times.

ELISA for TGF- β 1 and IL-6 in cell culture supernatants

Conditioned media were generated by plating approximately 3.0×10^5 ICC cells in 6-well dishes in growth medium overnight. The cells were washed 3 times with PBS, switched to serum-free medium (SFM). After incubation for indicated times, the supernatants were collected, and stored frozen at -80°C. TGF- β 1 and IL-6 concentrations were assessed by ELISA (DuoSet; R&D systems, Minneapolis, MN) according to the manufacturer's instructions. The plates were read on an ELN96 microplate reader (SCETI Co., Ltd., Tokyo, Japan) at 450 nm.

Experiments were also performed to assess the effects of recombinant human TGF- β 1 (rhTGF- β 1, R&D systems) and neutralizing anti-TGF- β 1 antibody on IL-6 production by the cell lines. Cells were cultured as describe above. SFM supplemented with rhTGF- β 1 (0, 1, 10 ng/mL, R&D systems), 5 μ g/mL neutralizing anti-TGF- β 1 antibody (mouse monoclonal, R&D systems), or nonimmune control IgG (mouse monoclonal IgG_{2B}, R&D systems) was collected after indicated times. IL-6 concentrations were

measured by ELISA by the method described above.

Western blotting

Approximately 3.0×10^5 HuCCT1 cells were cultured in growth medium overnight to determine the expression of the Smad2 phosphorylation (phospho-Smad2), p15^{Ink4B}, and plasminogen activator inhibitor-I (PAI-1). After 3 washes with PBS, the cells were switched to SFM with additional rhTGF- β 1, stimulated for indicated times, and lysed by M-PER protein extraction reagent (PIERCE Biotechnology, Rockford, IL) with Halt protease inhibitor cocktail (PIERCE Biotechnology) for protein extraction. Total cell lysates were electrophoresed by SDS-PAGE and transferred to membranes. Anti-Phospho-Smad2 (1:400; rabbit polyclonal, CHEMICON International, Temecula, CA), anti-p15^{Ink4B} antibody (1:200; rabbit polyclonal, Santa Cruz Biotechnology) and anti-PAI-1 antibody (1:500; rabbit polyclonal, Santa Cruz Biotechnology) was used together with a secondary horseradish peroxidase-conjugated polyclonal goat anti-rabbit antibody (1:1000; Medical & Biological Laboratories Co., Nagoya, Japan). Immunodetection was performed using an enhanced chemiluminescence kit for Western blotting detection (Amersham Biosciences, Buckinghamshire, UK). X-ray film was exposed for about 1 min for visualization. Autoradiograms of the immunoblots were scanned using Adobe Photoshop (Adobe System, Inc., San Jose, CA). The blots were adjusted for brightness and contrast for minimum background, and the mean density for each band was analyzed.

Real-time semi-quantitative PCR

Studies with cell lines were conducted to investigate the effects of rhTGF- β 1 and anti-TGF- β 1 antibodies on the expression of TGF- β 1, IL-6, p15^{Ink4B}, and PAI-1 mRNA. Conditioned media were generated by plating 3.0×10^5 ICC cells in 6-well dishes in growth medium overnight. The cells were washed 3 times with PBS, switched to SFM supplemented with rhTGF- β 1 (0, 0.1, 1, 10 ng/mL, 5 μ g/mL neutralizing anti-TGF- β 1 antibody, or 5 μ g/mL nonimmune control IgG, and incubated for indicated times. RNA was isolated using ISOGEN reagent (Nippon Gene Co., Toyama, Japan) according to the manufacturer's instructions. Total RNA (1 μ g per sample) was reverse transcribed in a final volume of 20 μ L using a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). TaqMan probes for human TGF- β 1, IL-6, p15^{Ink4B}, and PAI-1 RNA gene (TaqMan Gene Expression Assays; Applied Biosystems) were obtained. The samples were amplified in a total volume of 20 μ L containing 1 μ L of each specific probe and 10 μ L of the TaqMan PCR Core Reagent Kit (Universal PCR Master Mix; Applied Biosystems). The amplifications were performed in 40 cycles using a GeneAmp 5700 Sequence Detection system (Applied Biosystems), and the expression levels were calculated using the comparable cycle number method as recommended by the manufacturer. Semi-quantitative mRNA levels were evaluated based on the ratios of the mRNA levels of the target to those of the mRNA levels of GAPDH.

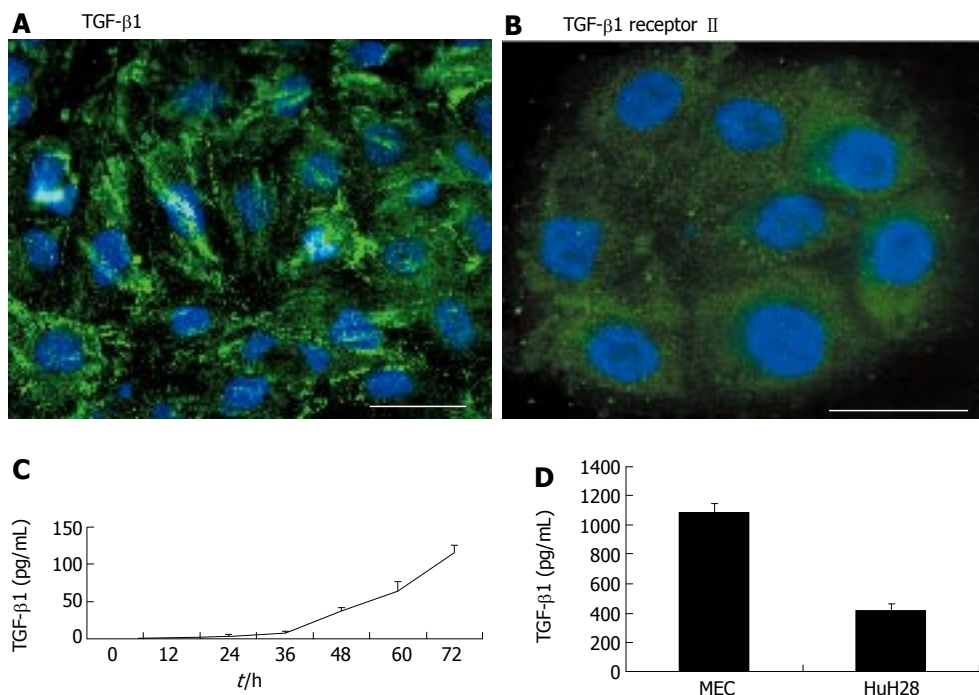


Figure 1 Expression of TGF-β1 and TGF-β receptor II in intrahepatic cholangiocarcinoma cell lines. Immunofluorescence showed staining of TGF-β1 (A) and TGF-β receptor II (B) in HuCCT1 cells. Scale bar = 20 μm. C: Detection of TGF-β1 in supernatant of HuCCT1 by ELISA. TGF-β1 levels in the supernatant of HuCCT1 showed a time-dependent increase; D: The other ICC cell lines were incubated for 48 h and assayed by ELISA to determine the levels of TGF-β1 in the supernatants. MEC and HuH-28 cells also secreted TGF-β1 into the supernatants.

³H-thymidine incorporation

1×10^4 cells were plated in a 96-well flat-bottomed plate in growth medium and changed to SFM 24 h later. The indicated concentrations of rhTGF-β1 and rhIL-6 (R&D systems) were added the following day for 12 h. For the measurement of DNA synthesis, 1 μCi/mL of ³H-thymidine (Perkin Elmer, Boston, MA) was added for 3 h. The ³H-thymidine incorporation was determined using a liquid scintillation counter (LSC-3000, Aloca, Tokyo, Japan) after detaching the cells from the plates with 0.25% trypsin.

Apoptosis assay

HuCCT1 and Mv1Lu cells were seeded on slide glass, and incubated in SFM in the absence or presence of rhTGF-β1 for 48 h. Apoptotic cells were identified by a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method. TUNEL assay was performed using an in situ Apoptosis Detection Kit (TAKARA BIO INC., Shiga, Japan) according to the manufacturer's instruction. Positive staining was detected by diaminobenzidine. Finally, slides were restained slightly with 1.0% methyl green. The apoptotic effect was determined by counting the number of positively labeled nuclei per 300 cells. The apoptotic index was calculated as follows: (number of apoptotic cells/total number counted) $\times 100\%$ ^[19].

RNA interference to IL-6Rα and STAT3

The mRNA expressions of interleukin-6 receptor (IL-6Rα or gp80) and the signal transducer and activator of transcription-3 (STAT3) were silenced by small interfering RNA (siRNA) using the SureSilencing siRNA kit (SuperArray Bioscience Co., Frederick, MD). One day before transfection, 5.0×10^4 cells were seeded in each well of a 24-well plate. The cells were transfected for 24 h at 37°C in a CO₂ incubator using transfection reagents

(Lipofectamine2000; Invitrogen Corp., Carlsbad, CA) and Opti-MEM Reduced-Serum Medium (GIBCO) according to the manufacturer's instructions. After transfection, the cells were incubated in SFM with or without 1 ng/mL TGF-β1 for 12 h, detached from the plates with 0.25% trypsin, and tested to determine DNA synthesis based on the method of ³H-thymidine incorporation described above. Reverse transcription-PCR (RT-PCR) was used to check the silencing effects of gene expression. The RT² real-time gene expression assay kit (SuperArray Bioscience Co.) and TaqMan probe (Applied Biosystems) were used for human IL-6Rα and STAT3, respectively.

Statistical analysis

The data were expressed as mean \pm SD ($n = 4$, independent experiments) and analyzed for significance using the Mann-Whitney *U*-test. A *P* value of less than .05 was considered statistically significant.

RESULTS

Expressions of TGF-β1 and TGF-β receptor II in human ICC

We began our study by confirming the expression of TGF-β1 and its receptors in the HuCCT1 cell line. Immunofluorescent staining with specific antibody confirmed the expression of TGF-β1 and TGF-β receptor II in HuCCT1 (Figure 1A and 1B). Next, we used the ELISA technique to examine the secretion of TGF-β1 from HuCCT1 into the medium. As shown in Figure 1C, HuCCT1 secreted TGF-β1 in a time-dependent fashion. The mean concentration of TGF-β1 in the supernatants after 72 h of incubation was 115.1 pg/mL. We also measured TGF-β1 production in two other ICC cell lines, MEC and HuH-28. TGF-β1 was secreted into the supernatants of the MEC and HuH-28 cell lines at mean concentrations of 1094.4 and 419.9 pg/mL at 48 h, respectively (Figure 1D).

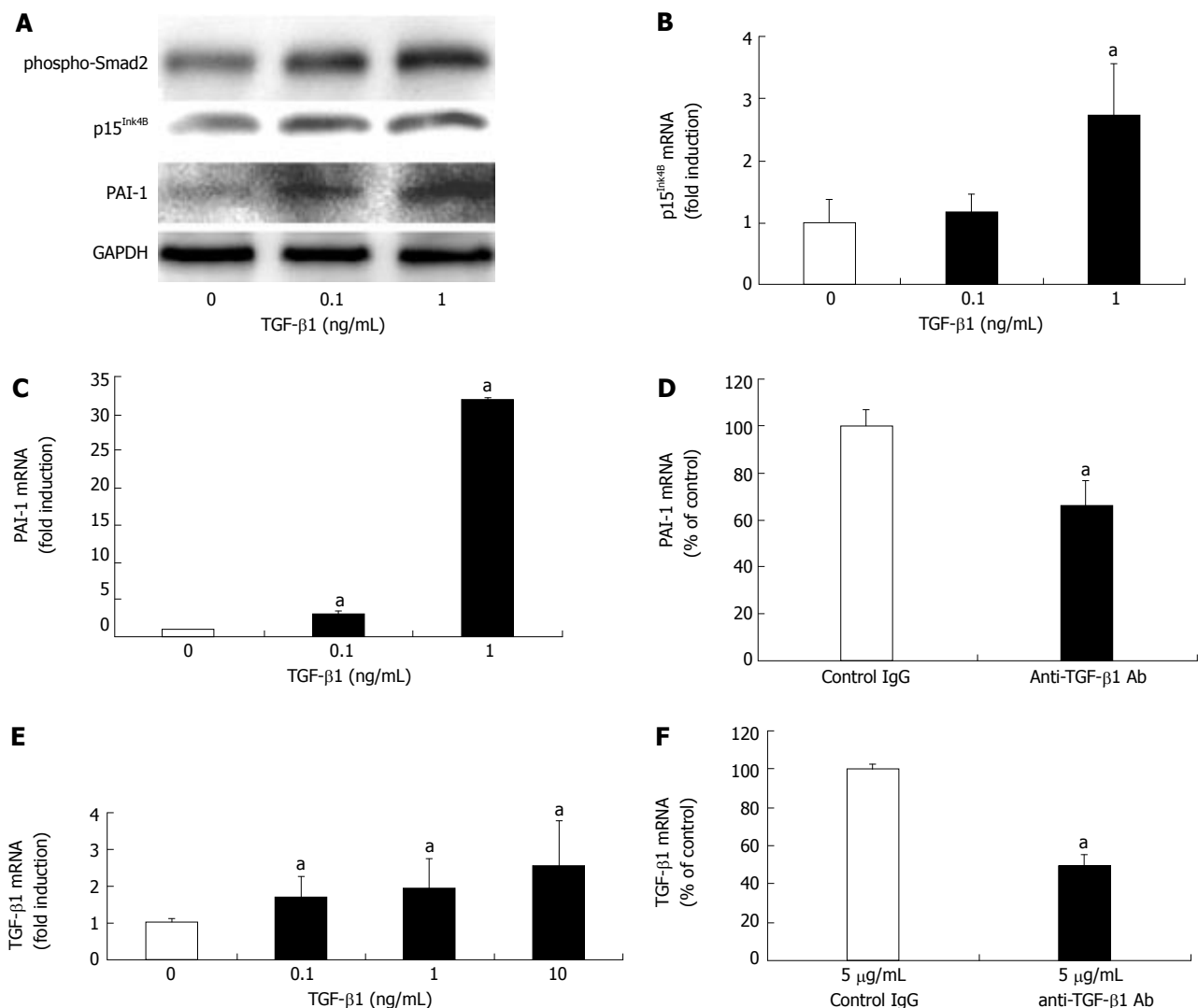


Figure 2 TGF- β 1 stimulates the TGF- β signaling pathway. **A:** The addition of 0.1 and 1 ng/mL TGF- β 1 augmented Smad2 phosphorylation (phospho-Smad2), p15^{Ink4B}, and PAI-1 proteins in Western blotting of HuCCT1 cells; **B:** TGF- β 1 stimulation for 48 h enhanced p15^{Ink4B} mRNA expression in HuCCT1 cells in RT-PCR: ^a $P < 0.05$ vs absence of TGF- β 1; **C:** TGF- β 1 stimulation for 48 h also augmented PAI-1 mRNA expression in HuCCT1 cells in RT-PCR: ^a $P < 0.05$ vs absence of TGF- β 1; **D:** PAI-1 mRNA was determined by RT-PCR after incubating the HuCCT1 cells with 5 ng/mL neutralizing anti-TGF- β 1 antibody or nonimmune control IgG for 96 h. The addition of anti-TGF- β 1 antibody attenuated the level of PAI-1 mRNA: ^a $P < 0.05$ vs control IgG; **E:** The addition of TGF- β 1 to HuCCT1 for 6 h led to a concentration-dependent increase in TGF- β 1 mRNA expression by RT-PCR: ^a $P < 0.05$ vs absence of TGF- β 1; **F:** TGF- β 1 mRNA in HuCCT1 was determined by RT-PCR after incubating the cells with 5 ng/mL neutralizing anti-TGF- β 1 antibody or nonimmune control IgG for 96 h. The addition of anti-TGF- β 1 antibody attenuated the level of TGF- β 1 mRNA: ^a $P < 0.05$ vs control IgG.

TGF- β 1 stimulates the TGF- β signaling pathway

TGF- β 1 has been reported to induce the expressions of p15^{Ink4B} and PAI-1 in a variety of different cells^[12,20]. Accordingly, we confirmed Smad2 phosphorylation (phospho-Smad2) and expression of the target genes p15^{Ink4B} and PAI-1 as markers of TGF- β 1 and the status of Smad-dependent signaling. Figure 2A demonstrates the effects of rhTGF- β 1 on endogenous expression of the phospho-Smad2, p15^{Ink4B}, and PAI-1 proteins in the HuCCT1 cell line. Western blotting of HuCCT1 cells after 48 h of stimulation by 0, 0.1, and 1 ng/mL rhTGF- β 1 revealed induction of phospho-Smad2, p15^{Ink4B}, and PAI-1 proteins. Similar results were obtained by measurements of the mRNA levels of p15^{Ink4B} and PAI-1. The expression of p15^{Ink4B} mRNA induced by 48 h of incubation with 1 ng/mL rhTGF- β 1 (Figure 2B) was significantly greater than that measured in the absence of rhTGF- β 1 stimulation. Forty-eight hours of incubation with rhTGF- β 1 at

concentrations of 0.1 and 1 ng/mL also induced significant increases in PAI-1 mRNA (3.0- and 32.0-fold increases, respectively) (Figure 2C). Furthermore, the addition of a neutralizing anti-TGF- β 1 antibody reduced the PAI-1 mRNA level. The addition of neutralizing TGF- β 1 antibody for 96 h significantly reduced PAI-1 mRNA to 66.1% of the level measured in the presence of nonimmune control IgG (Figure 2D). These findings demonstrate that secreted TGF- β 1 can stimulate the TGF- β signaling pathway mediated by Smads in an autocrine fashion without disrupting TGF- β signaling.

TGF- β 1 stimulates endogenous TGF- β 1 mRNA expression in an autocrine fashion

Next, we studied the effect of TGF- β 1 on endogenous TGF- β 1 mRNA expression in HuCCT1 with RT-PCR. Additional rhTGF- β 1 stimulation for 6 h resulted in a significant dose-dependent increase of TGF- β 1 mRNA

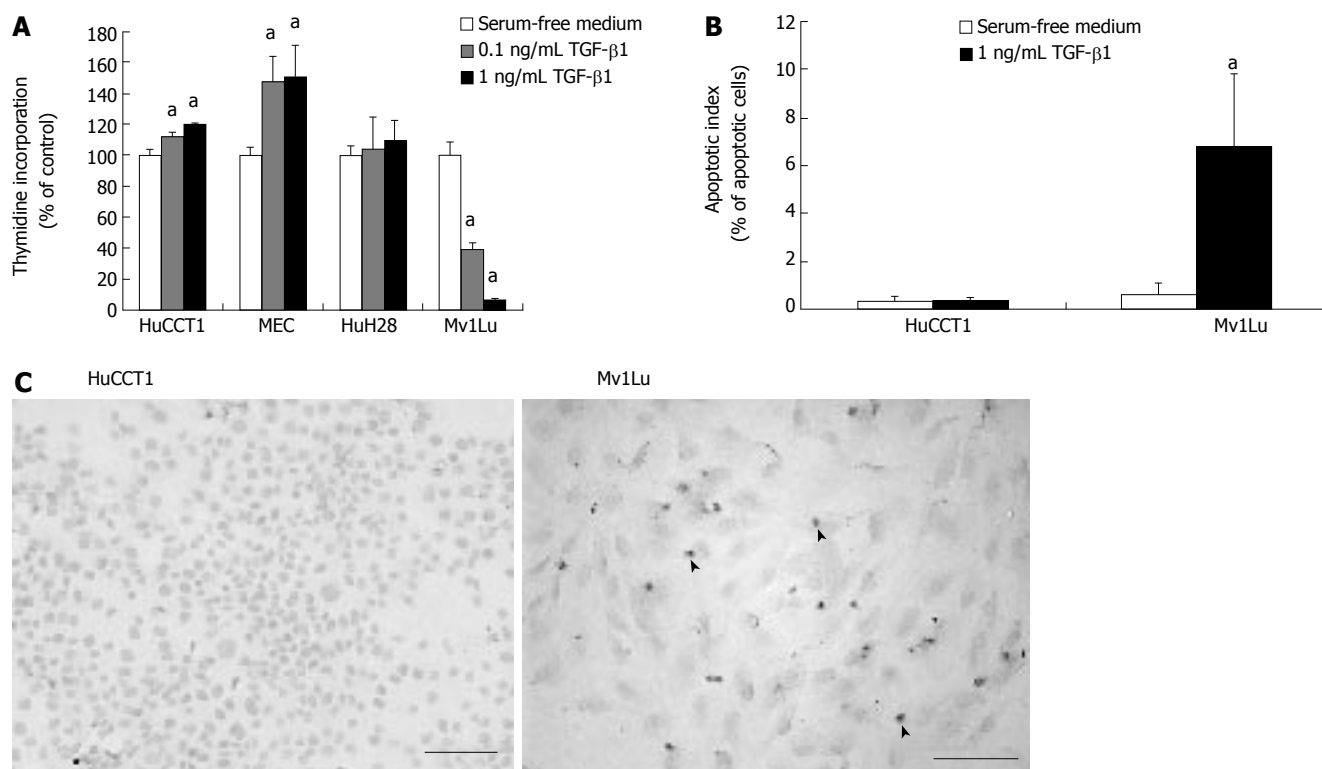


Figure 3 Effect of TGF-β1 on the cell growth and apoptosis of ICC cell lines. **A:** The cells were subjected to a ^3H -thymidine incorporation assay in the absence or presence of TGF-β1. No mitoinhibitory effect of TGF-β1 was observed in the ICC cells. TGF-β1 stimulation augmented the DNA synthesis of TGF-β1 in the HuCCT1 and MEC cells. In contrast, TGF-β1 treatment elicited a strong mitoinhibitory effect in Mv1Lu; **B:** Stimulation with 1 ng/mL TGF-β1 for 48 h did not induce apoptosis of HuCCT1 cells in a TUNEL assay. In the experiment using Mv1Lu, however, stimulation with TGF-β1 at the same concentration for the same duration did elicit an apoptotic effect: $^*P < 0.05$ vs absence of TGF-β1; **C:** HuCCT1 and Mv1Lu cells were treated with 1 ng/mL TGF-β1 for 48 h. The arrows denote apoptotic cells identified by the TUNEL method. Scale bar = 100 μm.

expression in HuCCT1 cells (Figure 2E). Moreover, the addition of neutralizing TGF-β1 antibody for 96 h resulted in a significant decrease of TGF-β1 mRNA to 49.1% of the level measured in the presence of nonimmune control IgG (Figure 2F).

TGF-β1 accelerates ICC cell proliferation

To further confirm the effect of TGF-β1 on ICC cells, we investigated whether TGF-β1 influenced the proliferation and apoptosis of ICC cells. The HuCCT1 and MEC cell lines showed significant increases in ^3H -thymidine incorporation in response to 0.1 and 1 ng/mL rhTGF-β1 stimulation for 12 h (Figure 3A). No mitoinhibitory effect of TGF-β1 was observed in any of the ICC cell lines in this study. In contrast, the additions of 0.1 and 1 ng/mL rhTGF-β1 to Mv1Lu resulted in 61.1% and 95.8% reductions in DNA synthesis, respectively.

Next, we applied the TUNEL method to determine whether we could observe apoptosis of the ICC cells. In an experiment using HuCCT1 cells, no significant differences in the levels of apoptosis were observed between cells incubated in SFM for 48 h and cells incubated with 1 ng/mL rhTGF-β1 for 48 h. However, the TUNEL assay demonstrated that 1 ng/mL rhTGF-β1 induced evident apoptosis of Mv1Lu cells, and the apoptotic index at 48 h after rhTGF-β1 treatment was significantly higher than that of the control group (Figure 3B). Almost no apoptotic cells were observed in the HuCCT1 cells stimulated with 1 ng/mL rhTGF-β1 for 48

h (Figure 3C). However, the TUNEL assay using Mv1Lu indicated an increase of apoptotic cells in response to 1 ng/mL rhTGF-β1 for 48 h. On the basis of these results, we can conclude that TGF-β1 inhibits cell proliferation and induces apoptosis in normal epithelial cells, whereas ICC cells are equipped with a mechanism to resist the growth-inhibitory response and apoptotic effect induced by TGF-β1.

TGF-β1 stimulates the secretion of IL-6

Previous studies have shown that IL-6 can induce the proliferation of ICC cells^[21,22]. To elucidate the mechanism further, we measured the level of IL-6 production by ICC cells and explored the relationship between TGF-β1 and IL-6. A time-dependent increase of IL-6 was observed in the HuCCT1 supernatants unexposed to TGF-β1. To investigate the potential interaction between TGF-β1 and IL-6, we evaluated the levels of IL-6 in the HuCCT1 supernatants stimulated with rhTGF-β1. As a result, we found a time-dependent increase of IL-6 concentrations in response to rhTGF-β1 throughout the 72-h observation period (Figure 4A). The increases in IL-6 induced by 1 and 10 ng/mL rhTGF-β1 after 72 h of incubation (2.2- and 5.7-fold increases, respectively) were significantly greater than the increases observed in the supernatants from cells untreated by rhTGF-β1. Similar results were obtained in IL-6 mRNA quantification after 6 h of stimulation by rhTGF-β1. A significant 2.7-fold increase of IL-6 mRNA was observed in the HuCCT1 cells stimulated with 10

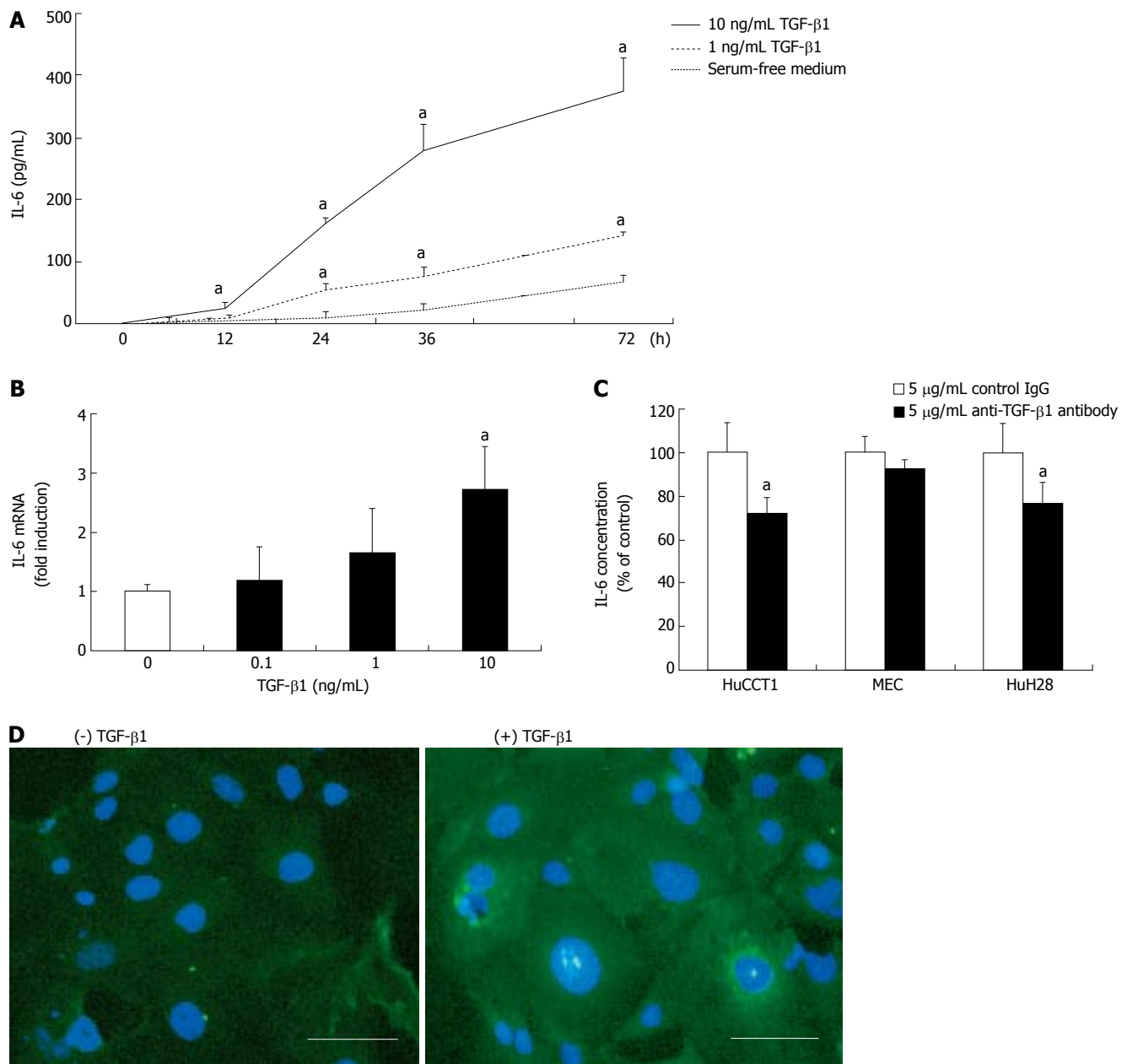


Figure 4 Effect of TGF- β 1 on the IL-6 expression of ICC cells. **A:** ELISA revealed the secretion of IL-6 by HuCCT1 into the serum-free medium. HuCCT1 also showed a time-dependent increase of IL-6 concentrations in response to 1 and 10 ng/mL TGF- β 1 stimulation: $^aP < 0.05$ vs absence of TGF- β 1; **B:** The addition of TGF- β 1 to HuCCT1 for 6 h resulted in a dose-dependent increase of IL-6 mRNA expression by RT-PCR: $^aP < 0.05$ vs absence of TGF- β 1; **C:** IL-6 levels in the supernatants were determined by ELISA after incubating ICC cells with 5 ng/mL neutralizing anti-TGF- β 1 antibody or nonimmune control IgG for 96 h. The addition of neutralizing anti-TGF- β 1 antibody resulted in a decrease of IL-6 production in HuCCT1 and HuH28: $^aP < 0.05$ vs control IgG; **D:** An immunofluorescence study using HuCCT1 cells demonstrated an increase in IL-6 production in response to TGF- β 1. Immunoreactivity with a specific anti-IL6 antibody was weak, with no stimulation of TGF- β 1, whereas 10 ng/mL TGF- β 1 induced IL-6 expression in the cytoplasm of the HuCCT1 cells. Scale bar = 20 μ m.

ng/mL TGF- β 1 (Figure 4B). IL-6 production was also confirmed in the MEC and HuH-28 ICC cell lines. After 72 h of incubation in SFM, the mean IL-6 concentrations in the supernatants of MEC and HuH-28 cells were 307.4 and 6.93 ng/mL, respectively. We next investigated the effects of a neutralizing anti-TGF- β 1 antibody on IL-6 production in ICC cell supernatants. Compared with nonimmune control IgG, the addition of anti-TGF- β 1 antibody resulted in a significant inhibition of IL-6 concentrations in the HuCCT1 and HuH28 supernatants (Figure 4C). Next, we investigated the IL-6 localization in response to rhTGF- β 1 by immunofluorescence. As shown

in Figure 4D, the cytoplasm of HuCCT1 cells untreated by rhTGF- β 1 was only weakly immunoreactive to the anti-IL-6 antibody. In contrast, we identified a dominant localization of IL-6 in the cytoplasm of the HuCCT1 cells stimulated with 10 ng/mL rhTGF- β 1 for 48 h.

Effects of TGF- β 1 on ICC cell growth under inhibition of IL-6 functions via RNA interference with IL-6R α (gp80) and STAT3

We measured thymidine incorporation in the ICC cells after the addition of 0.1 and 1 ng/mL rhIL-6 to clarify whether IL-6 accelerates the cell growth of ICC cells.

As seen in Figure 5A, rhIL-6 brought about significant increases in thymidine incorporation in all of the ICC cells studied. This, taken together with the results of the other experiments described in this study, indicates that TGF- β 1 and IL-6 act as ICC growth factors with interrelated functions.

To further elucidate the mechanism of TGF- β 1-induced proliferation of ICC, we examined whether TGF- β 1 functioned as a growth factor while IL-6 functions were inhibited with the use of small interfering RNA (siRNA) in order to silence the expression of IL-6R α and STAT3 mRNA. As shown in Figure 5B, siRNA treatment suppressed IL-6R α and STAT3 mRNA expressions in the HuCCT1 cells to 10.6% and 41.9% of the control siRNA level, respectively. Figure 5C shows the effects of rhTGF- β 1 on DNA synthesis in HuCCT1 cells with siRNA against IL-6R α and STAT3. HuCCT1 stimulated by 1 ng/mL rhTGF- β 1 exhibited a 37.4% increase of DNA synthesis under the control siRNA condition, whereas HuCCT1 stimulated by rhTGF- β 1 during siRNA silencing of IL-6R α brought about no increase after TGF- β 1 stimulation. Furthermore, HuCCT1 stimulated by TGF- β 1 during siRNA silencing of STAT3 exhibited a 19.4% decrease of DNA synthesis. These findings indicate that the TGF- β 1-induced production of IL-6 confers resistance to the growth inhibition by TGF- β 1 and plays a role in the mechanism of TGF- β 1-induced ICC proliferation.

DISCUSSION

In previous studies, ICC cells were found to resist the growth inhibitory effect of TGF- β 1^[18,22]. Our current study, in contrast, demonstrated that TGF- β 1 stimulation in ICC resulted in cellular proliferation rather than resistance to the innate mitoinhibition. The molecular mechanism by which some malignant cells evade the mitoinhibitory effect of TGF- β 1 remains obscure. Recent studies on malignancies have demonstrated the disruption of TGF- β signaling, including lack or mutations of the TGF- β receptor^[23-26] and the Smad family^[26-29]. This study, in contrast, revealed no disruption of TGF- β signaling. In fact, TGF- β 1 was found to activate the expression of PAI-1 as a target gene via Smad2 phosphorylation. These results suggest that ICC cells are equipped with a mechanism to resist the innate mitoinhibitory response and encourage cell activation by TGF- β 1. Given that TGF- β 1 upregulation has been identified in both pancreatic cancer and hepatocellular carcinoma, we know that it may also contribute to disease progression in patients with some malignancies^[12,30,31].

The results from our current study demonstrate that the growth effect of TGF- β 1 in ICC cells is closely associated with IL-6 production and function. IL-6 plays a key role in the hepatic response to inflammation, as well as in the regulation of bile duct growth^[32-35]. ICC is generally characterized by strong cellular proliferation and inflammation around the tumor. From these points of view, we decided to perform several studies on IL-6, a reported inducer of ICC growth^[21,22,36,37]. IL-6 is elevated in the serum of patients with ICC^[38] and expressed in

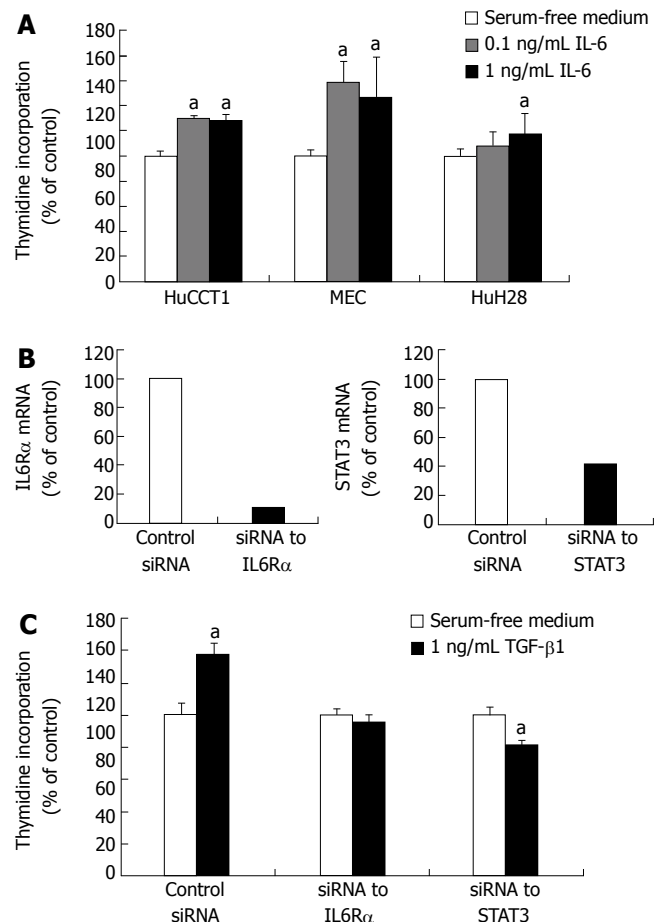


Figure 5 Effects of TGF- β 1 on DNA synthesis during inhibition of IL-6 functions. **A:** All of the ICC cells demonstrated enhanced DNA synthesis in response to IL-6: ^a $P < 0.05$ vs absence of IL-6. We next investigated whether TGF- β 1 promoted ICC cell growth during the inhibition of IL-6 functions by small interfering RNA (siRNA); **B:** RT-PCR was conducted to assess the effects of siRNA in suppressing the mRNA expressions of IL-6R α and STAT3. IL-6R α and STAT3 mRNA expression was suppressed by 10.6% and 41.9%, relative to control siRNA; **C:** The silencing of IL-6R α and STAT3 mRNA expressions by siRNA in HuCCT1 cells led to a suppression in the TGF- β 1-induced DNA synthesis relative to that observed under the control siRNA condition: ^a $P < 0.05$ vs absence of rhTGF- β 1.

ICC surgical specimens and cell lines^[21,36,39]. All of the ICC cell lines used in our study expressed IL-6. This IL-6 expression was augmented by TGF- β 1 and attenuated by the neutralizing anti-TGF- β 1 antibody. A similar mechanism of TGF- β 1-induced IL-6 expression was also reported in prostate cancer cells^[40]. Stimulation by IL-6 augmented DNA synthesis in all cell lines. In contrast, TGF- β 1 stimulation during siRNA silencing of IL-6R α and STAT3 mRNA expression led decreased levels of DNA synthesis in HuCCT1 cells. On this basis, we know that the two cytokines share a functional relation, and further, that IL-6 function is very important for the TGF- β 1-based growth effect in ICC. Recent studies on ICC cells have also demonstrated effects of TGF- β 1 on the induction of prostaglandin E₂^[41] and vascular epithelial growth factor (VEGF)^[17], two factors closely related to tumor proliferation and angiogenesis. This may shed light on the oncogenic switch to resist the innate growth inhibitory effect of TGF- β 1. As one step in this switch, the malignant cells might acquire a mechanism to produce

cell growth inducers via the stimulation of TGF- β 1.

IL-6 has also been reported to participate in the chemoresistance of IL-6-induced inhibitors of apoptosis protein against conventional chemotherapeutic agents^[42]. In addition to facilitating ICC cell growth, IL-6 aggravates the difficulties in treating malignancy. Effective regulators of these cytokines might be attractive therapeutic targets to encourage tumor cell apoptosis and cell growth inhibition.

In summary, we have provided evidence in support of the autocrine growth effect of TGF- β 1 in human ICC. We also demonstrated that the TGF- β 1-induced growth effect is closely associated with IL-6 function. ICC has acquired these mechanisms to resist the well-known growth inhibitory response of TGF- β 1. Further elucidation of the TGF- β 1 and IL-6 function in cholangiocarcinogenesis may afford an important opportunity to define a novel molecular target for ICC prevention and treatment.

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Impact of lipoprotein lipase gene polymorphisms on ulcerative colitis

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Abstract

AIM: To examine the influence of lipoprotein lipase (LPL) gene polymorphism in ulcerative colitis (UC) patients.

METHODS: Peripheral blood was obtained from 131 patients with UC and 106 healthy controls for DNA extraction. We determined LPL gene polymorphisms affecting the enzyme at Ser447stop, as well as *Hind*III and *Pvu*II polymorphisms using PCR techniques. PCR products were characterized by PCR-RFLP and direct sequencing. Polymorphisms were examined for association with clinical features in UC patients. Genotype frequencies for LPL polymorphisms were also compared between UC patients and controls.

RESULTS: In patients with onset at age 20 years or younger, C/G and G/G genotypes for Ser447stop polymorphism were more prevalent than C/C genotype (OR = 3.13, 95% CI = 0.95-10.33). Patients with H^{+/+} or H^{-/-}

genotype for *Hind*III polymorphism also were more numerous than those with H^{+/+} genotype (OR = 2.51, 95% CI = 0.85-7.45). In the group with H^{+/+} genotype for *Hind*III polymorphism, more patients had serum triglyceride concentrations over 150 mg/dL than patients with H^{+/+} or H^{-/-} genotype (*P* < 0.01, OR = 6.46, 95% CI = 1.39-30.12). Hypertriglyceremia was also more prevalent in patients with P^{+/+} genotypes for *Pvu*II polymorphism (*P* < 0.05, OR = 3.0, 95% CI = 1.06-8.50). Genotype frequency for LPL polymorphism did not differ significantly between UC patients and controls.

CONCLUSION: Ser447stop and *Hind*III LPL polymorphisms may influence age of onset of UC, while *Hind*III and *Pvu*II polymorphisms influence serum triglyceride in UC patients.

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Key words: Ulcerative colitis; Lipoprotein lipase; Lipid metabolism; Triglyceride

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INTRODUCTION

Inflammatory bowel disease (IBD), characterized by chronic recurrent inflammation of the intestinal tract, includes two common forms: Crohn's disease (CD) and ulcerative colitis (UC). For both forms, the etiology is unclear, but likely to be multifactorial. Factors that may affect IBD include diet, infantile environment and immune defense abnormalities limited to the intestinal tract. Recently, genetic factors have been examined in IBD, but many studies seeking susceptibility genes for IBD have not produced a consensus. We presently investigated possible influence of the lipoprotein lipase (LPL) gene in UC. LPL plays a critical role in lipid metabolism. Dietary triacylglycerol (TAG) exists in the human circulation as macromol-

ecules (TAG-rich lipoprotein) that are too large to pass through the endothelium of most capillaries. LPL catalyses conversion of TAG-rich lipoprotein to triglyceride (TG), very low-density lipoprotein (VLDL), and chylomicrons (CM), all of which circulate and can enter tissues more readily to serve energy source^[1,2]. Our interest in LPL in IBD was provoked by identifying high lipid intake as a risk factor for IBD. Shoda *et al*^[3] reported associated intake of n-6 polyunsaturated fat and animal fat with development of CD in Japanese patients, while Geering *et al*^[4] reported high intake of mono- and polyunsaturated fats to be a risk factor for the UC. Furthermore, the LPL gene has been localized to chromosome 8p22 near N-acetyltransferase2 (NAT2) gene, while Machida *et al*^[5] reported an association between NAT2 gene haplotype NAT2*7B and CD. Considering these various findings, we suspected that LPL gene polymorphisms could influence characteristics and incidence of UC. These issues were examined in the present molecular genetic investigation.

MATERIALS AND METHODS

Subjects

We studied 131 patients with UC (75 males and 56 females) and 106 healthy controls (53 males and 53 females). Diagnosis of UC was based on conventional clinical, radiologic, endoscopic, and pathologic criteria. Characteristics of UC patients are shown in Table 1. We investigated the effect of LPL polymorphism on clinical features as shown in this table. To examine LPL polymorphisms in terms of influence on UC incidence, we compared LPL genotype frequencies in UC patients with those in controls.

DNA extraction

Blood samples were obtained from patients and controls after they had given informed consent to sampling and analyses. This study was approved by the Fujita Health University Ethics Committee. DNA was extracted from blood samples using a PUREGENE DNA isolation kit (Gentra Systems, Inc, Minneapolis, USA).

Genotype

LPL polymorphisms were typed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods to detect Ser447stop, *Hind*III and *Pvu*II polymorphisms^[6]. The primer sets were as follows: for Ser447stop, 5'-GATGTGGCCTGAGTGTGACAG-3' (forward) and 5'-TCCCTTAGGGTGCAAGCTCAG-3' (reverse); for *Hind*III, 5'-GATGTCTACCTGGATAATCAAAG-3' (forward) and 5'-CTTCAGCTAGACATTGCTAGTGT-3' (reverse); and for *Pvu*II, 5'-GAGACACAGATCTCTCTTAAGAC-3' (forward) and 5'-ATCAGGCAATGCGTATGAGGTAA-3' (reverse). PCR was carried out in a 30-μL aliquot containing 50 ng of genomic DNA, 12 pmol of each primer, 3.0 μL of 10 × buffer solution, 20 nmol/μL of dNTP, and 1U of Taq polymerase. PCR conditions for Ser447stop included initial denaturation at 95°C for 5 min, followed by 35 amplifica-

Table 1 Characteristics of patients with ulcerative colitis

Characteristic	UC (n = 131)
Age of onset (yr)	36.8 ± 15.3
Colitis duration (yr)	8.8 ± 7.7
Extension	
Proctitis	22 (16.8%)
Left-sided	59 (45.0%)
Pancolitis	50 (38.2%)
Type of clinical course	
First episode	14 (10.7%)
Chronic relapse	79 (60.3%)
Chronic persistent	38 (29.0%)
Severity	
Mild	40 (30.5%)
Moderate	61 (46.6%)
Severe	30 (22.9%)

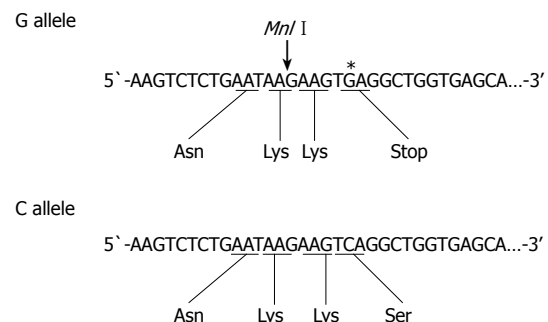


Figure 1 Strategy for detection of the G/C allele (Ser/stop) in exon 9 of the lipoprotein lipase by PCR-RFLP analysis. Introduction of G (*) is in place of C creates a new *Mnl* I site.

tion cycles, each cycle containing denaturation at 95°C for 30 s, primer annealing at 62°C for 30 s, and extension at 72°C for 30 s. PCR conditions for *Hind*III and *Pvu*II included initial denaturation at 95°C for 5min, followed by 35 amplification cycles, each cycle containing denaturation at 95°C for 30 s, primer annealing at 61°C for 30 s, and extension at 72°C for 30 s. PCR products subjected to overnight digestion with *Mnl*I, *Hind*III, and *Pvu*II were examined by electrophoresis on 20 g/L agarose gels. The strategy for the detection of the G allele (Ser447stop) is shown in Figure 1. A 315-bp segment of exon 9 of the lipoprotein lipase gene was amplified by the PCR. In the presence of the G allele, the amplified DNA contains a new site for *Mnl*I, but does not contain a site in the presence of the C allele. However, there is a restriction site for *Mnl*I in the forward primer sequence regardless of G/C mutation. After digestion, the PCR product yielded 248- and 67-bp fragments. The PCR product containing a *Hind*III restriction site yielded 210- and 140-bp fragments, while the product containing a *Pvu*II restriction site yielded 222- and 209-bp fragments. Some products after digestion were examined on polyacrylamide gels (GeneGel Excel 12.5/24 kit from GE Healthcare Bio-sciences, Tokyo) and stained with DNA silver staining kit (GE Healthcare Bio-sciences,

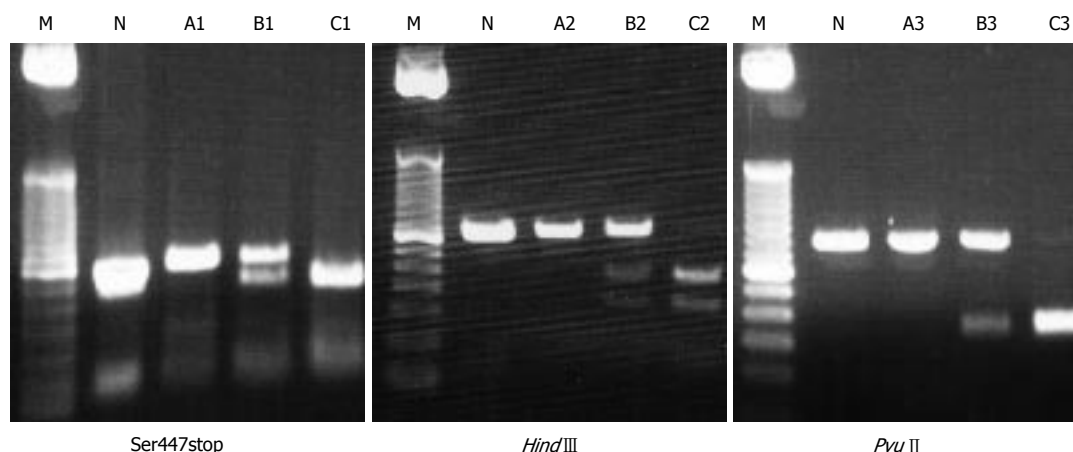


Figure 2 Electrophoresed pattern of LPL polymorphism. M: Marker, N: No digestion. In Ser447stop, A1: C/C genotype (315 bp); B1: C/G genotype; C1: G/G genotype. In *Hind*III, A2: H^{+/+} genotype; B2: H^{+/-} genotype (350 bp); C2: H^{-/-} genotype. In *Pvu*II, A3: P^{+/+} genotype (431 bp); B3: P^{+/-} genotype; C3: P^{-/-} genotype.

Table 2 Allele frequency and genotype distribution of LPL polymorphism in UC patients and controls

	Allele frequency		Genotype (%)		OR (95% CI)
Ser447stop	C	G	CC	CG+GG	
UC (n = 131)	0.92	0.08	111 (84.7)	19 + 1 (15.3)	1.49
Control (n = 104)	0.88	0.12	82 (78.8)	19 + 3 (21.2)	(0.76-2.91)
<i>Hind</i> III	H ⁺	H ⁻	H ^{+/+}	H ^{+/-} + H ^{-/-}	
UC (n = 130)	0.80	0.20	86 (66.1)	37 + 7 (33.9)	1.28
Control (n = 106)	0.74	0.26	64 (60.4)	28 + 14 (39.6)	(0.75-2.18)
<i>Pvu</i> II	P ⁺	P ⁻	P ^{+/+}	P ^{+/-} + P ^{-/-}	
UC (n = 131)	0.70	0.30	65 (49.6)	53 + 13 (50.4)	1.44
Control (n = 106)	0.64	0.36	43 (40.6)	50 + 13 (59.4)	(0.86-2.42)

Table 3 Age of onset of UC patients and LPL polymorphisms

Age of onset (yr)	Genotype		OR (95% CI)
Ser447stop	C/G+G/G	C/C	
< 20 (n = 15)	5	10	3.13
≥ 20 (n = 116)	16	100	(0.94-10.33)
<i>Hind</i> III	H ^{+/+} + H ^{+/-}	H ^{+/+}	
< 20 (n = 15)	8	7	2.31
≥ 20 (n = 115)	36	79	(0.84-7.45)
<i>Pvu</i> II	P ^{+/+} + P ^{+/-}	P ^{+/+}	
< 20 (n = 15)	9	6	1.55
≥ 20 (n = 116)	57	59	(0.52-4.64)

Tokyo), e.g. *Pvu*II digestion-cases. Electrophoresed patterns of LPL polymorphisms are shown in Figure 2.

Results of PCR-RFLP were confirmed by direct sequencing. DNA was extracted from agarose gels using an extraction kit (QIAGEN, Hilden, Germany). Then genotype was confirmed by sequence analysis using an auto sequencer (data not shown).

Statistical analysis

All data were analyzed by Excel 2000 and STASTISCA software. Clinical features of UC, allele frequency, and genotype distribution were evaluated by using χ^2 test. A *P* value less than 0.05 was considered statistically significant.

Allelelic and genotype frequencies were determined from observed genotype counts, and the extensions of Hardy-Weinberg equilibrium were evaluated by using χ^2 test.

RESULTS

LPL polymorphisms and risk of UC

The allele frequency of LPL polymorphism and the genotype distribution are shown in Table 2. The observed genotype data were consistent with Hardy-Weinberg equilibrium (Ser447stop: $\chi^2 = 0.48$, *P* = 0.49; *Hind*III: $\chi^2 = 1.73$, *P* = 0.19; *Pvu*II: $\chi^2 = 0.88$, *P* = 0.35). In the patient group, one patient was not able to identify with genotype of *Hind*III polymorphism. In the control group, two patients

were not able to identify with genotype of Ser447stop polymorphism. The allele frequency of Ser447stop polymorphism showed no difference between UC patients and controls. Frequencies of C/C, C/G and G/G genotypes of Ser447stop polymorphism in the control group were 78.8%, 18.3% and 2.9%, respectively, while these were not significantly different in patients. In the control group, frequencies of H^{+/+}, H^{+/-} and H^{-/-} genotypes for *Hind*III polymorphism were 60.4%, 26.4% and 13.2%, respectively, while frequencies of P^{+/+}, P^{+/-} and P^{-/-} genotype for *Pvu*II polymorphism were 40.6%, 47.1% and 12.3%, respectively. In UC patients, neither *Hind*III nor *Pvu*II polymorphisms differed significantly from genotype and allele frequencies in controls.

LPL polymorphism and characteristics of UC patients

We sought to identify associations between characteristics of UC patients (age at onset, gender, nature of the clinical course, extent of lesions, severity of colitis) and LPL polymorphisms. The relationship between age of onset of UC patients and their LPL polymorphisms are summarized in Table 3. In patients with onset at age 20 years or younger, more patients had either C/G or G/G genotype for Ser447stop than a C/C genotype (OR = 3.13, 95% CI = 0.95-10.33). In this early-onset group, more patients had either an H^{+/+} or H^{+/-} genotype for *Hind*III polymorphism than an H^{+/+} genotype (OR = 2.51, 95% CI = 0.85-7.45). Other characteristics (gender, nature of

Table 4 Triglyceride levels of UC patients and LPL polymorphisms

Triglycerides (mg/dL)	Genotype		OR (95%CI)
	C/C	C/G + G/G	
Ser447stop			
≥ 150 (n = 20)	19	1	4.93 (0.61-40.03)
< 150 (n = 68)	54	14	
<i>Hind</i> III	H ^{+/+}	H ^{+/-} + H ^{-/-}	
≥ 150 (n = 20)	18 ^b	2	6.46 (1.39-30.12)
< 150 (n = 67)	39	28	
<i>Pvu</i> II	P ^{+/+}	P ^{+/-} + P ^{-/-}	
≥ 150 (n = 20)	13 ^a	7	3.0 (1.06-8.50)
< 150 (n = 68)	26	42	

^aP < 0.05 vs P^{+/-} + P^{-/-} group; ^bP < 0.01 vs H^{+/-} + H^{-/-} group.

the clinical course, extent of lesions, severity of colitis) showed no significant differences between polymorphism-defined groups. Details of treatment among UC patients contained 5-aminosalicylic acid agents only (35 cases), steroid therapy (65 cases), operation (20 cases), other therapy (plasma exchange in 31 cases, immunosuppressant in 4 cases). We classified UC patients into a steroid-effective group (remission with a conventional steroid dose) and a steroid-resistant group (lack of such a remission, requiring surgery or other therapy, such as plasma exchange). We did not find an effect of LPL polymorphism on steroid effectiveness of UC patients.

We also investigated serum total cholesterol in terms of LPL polymorphism in UC patients. First, 108 UC patients were classified into groups with total cholesterol below or above 220 mg/dL. LPL polymorphisms did not differ between these groups. Next, triglyceride concentration in 88 UC patients was divided into those below or above 150 mg/dL (Table 4). Patients with an H^{+/+} genotype for *Hind*III polymorphism were more likely to have triglyceride concentrations over 150 mg/dL than those with an H^{+/-} or H^{-/-} genotype (P < 0.01, OR = 6.46, 95% CI = 1.39-30.12). This was also true for patients with a P^{+/+} genotype for *Pvu* II polymorphism compared with those with P^{+/-} or P^{-/-} genotype (P < 0.05, OR = 3.0, 95% CI = 1.06-8.50).

DISCUSSION

The LPL gene, located on chromosome 8p22^[7], consists of 10 exons. As for the LPL polymorphisms investigated in this study, the Ser447stop polymorphism is within exon 9^[8], involving substitution of G for C at nucleotide 1595. The *Hind*III polymorphism is located in intron 8^[9,10], and *Pvu* II in intron 6^[9,11]. Abnormalities in LPL function have been associated with various diseases, those linked with LPL polymorphisms are cardiovascular disease^[12], cerebrovascular disease^[13], chylomycronemia^[14], insulin resistance^[15], Alzheimer's disease^[16] and various infections^[17]. Life styles and habits also can influence many diseases, possibly including UC. We presently sought out the relationship between UC and the LPL gene.

This study is probably the first to report an association

between characteristic of UC and LPL polymorphism, representing two important findings. The first finding was that *Hind*III and *Pvu* II polymorphisms affected serum triglyceride concentrations in UC patients. Some studies reported that Ser447stop polymorphism activated LPL, decreasing triglycerides and increasing HDL cholesterol. As opposed to Ser447stop, *Hind*III polymorphism lowered LPL activity, and elevated triglycerides, and decreased HDL cholesterol. *Pvu* II polymorphism has similar function^[12,18]. Using a cutoff triglyceride value of 150 mg/dL, the above relationship for *Hind*III and *Pvu* II polymorphisms held true in our UC patients. Patients homozygous for *Hind*III polymorphism were even more likely to have triglyceride above 150 mg/dL than those homozygous for *Pvu* II polymorphism. *Hind*III polymorphism, therefore, would appear to suppress LPL activity more strongly than *Pvu* II polymorphism. Among studies of associations between triglyceride in healthy individuals and LPL polymorphism, Ann *et al*^[19] reported that *Hind*III polymorphism elevated triglycerides, while Chamberlain *et al*^[18] pointed out some subjects with *Hind*III polymorphism showed normal concentrations. Since associations between elevated triglyceride in healthy individuals and *Hind*III polymorphism thus are a matter of some disagreement, our finding that UC patients with H^{+/+} genotype for LPL polymorphism were particularly likely to have elevated triglycerides, some part of the difference seen in our UC patients might be specific to UC.

Previous studies have reported that serum triglyceride concentrations did not differ between UC patients and controls^[20,21]. In this study, however, patients with H^{+/+} genotype had serum triglyceride concentrations over 150 mg/dL compared to patients with H^{+/-} or H^{-/-}, thereby suggesting that *Hind*III polymorphism may contribute to elevate triglyceride levels directly or may influence other gene-elevated triglyceride concentrations. Although few studies have examined associations between UC and LPL, lipid intake has been reported to influence risk of IBD. Many studies have reported relationship between lipid metabolism and inflammation. During various human inflammatory states, serum triglyceride concentrations increase because some cytokines responsible for inflammatory responses, including tumor necrosis factor (TNF)-α, interferon (IFN)-γ, inhibit LPL activity^[22]. Other studies reported that lipopolysaccharides derived from Gram-negative bacteria reduced LPL activity in macrophages^[23]. LPL also directly induces the expression of the TNF-α gene^[24], which synergizes with IFN-γ in stimulating nitric oxide synthetase expression in macrophages^[25]. Since LPL is strongly associated with cytokines, it may contribute to development of inflammation. In addition, De Sanctis *et al*^[26] reported an association between natural killer (NK) cells and LPL polymorphism. Some studies have suggested that UC is associated with changes in humoral immunity^[27,28] and cellular immunity^[29], while UC may be associated with loss of immune tolerance in the intestine. Association between LPL and characteristic of UC may shed some light on mechanisms of onset of UC.

The second finding was that Ser447stop polymorphism

and *Hind*III polymorphism might be associated with age of onset of UC patients. In patients with onset at 20 years or younger, more patients had either C/G or G/G genotype at Ser447stop than a C/C genotype, while more patients had either H^{+/+} or H^{-/-} genotype for *Hind*III polymorphism than H^{+/+} genotype. As for the significance of relatively early onset, the LPL gene might influence onset of disease directly or by acting upon another gene or factor. In addition to genotype, age may affect LPL activity, while lipid metabolism might influence onset of IBD. Hamilton *et al.*^[30] reported that older individuals had inefficient triglyceride metabolism and elevated serum triglycerides because of reduction of LPL activity in postural skeletal muscle during aging. Among UC patients in our study, 10% (2/20) of those aged 20 to 29 years and 15% (4/26) of those aged 30 to 39 years showed triglycerides over 150 mg/dL, while Arai *et al.*^[31] reported that, in a Japanese population sample, the mean triglyceride concentration was 83 mg/dL in the third, and 118 mg/dL in the fourth decade of life. Our finding that younger UC patients appeared more likely to have high triglyceride concentration requires further investigation.

In conclusion, our study indicates that LPL polymorphism influences lipid metabolism in UC patients and age of onset of UC, and might contribute to onset and biologic behavior of UC.

Many studies have considered gene polymorphisms in UC, but few have suggested an influence of polymorphism on metabolism in UC patients. Further study on LPL and other gene polymorphisms in UC remains an important line of investigation.

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Natural history of a randomized trial comparing distal spleno-renal shunt with endoscopic sclerotherapy in the prevention of variceal rebleeding: A lesson from the past

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Abstract

AIM: To compare endoscopic sclerotherapy (ES) with distal spleno-renal shunt (DSRS) in the prevention of recurrent variceal bleeding in cirrhotic patients during a long-term follow-up period.

METHODS: In 1984 we started a prospective, controlled study of patients with liver cirrhosis. Long-term follow-up presents a natural history of liver cirrhosis complicated by advanced portal hypertension. In this study the effects of 2 types of treatment, DSRS or ES, were evaluated. The study population included 80 patients with cirrhosis and portal hypertension referred to our department from October 1984 to March 1991. These patients were drawn from a pool of 282 patients who underwent either elective surgery or ES during the same period of time. Patients were assigned to one of the 2 groups according to a random number table: 40 to DSRS and 40 to ES using polidocanol.

RESULTS: During the postoperative period, no DSRS patient died, while one ES patient died of uncontrolled hemorrhage. One DSRS patient had mild recurrent variceal hemorrhage despite an angiographically patent DSRS and another patient suffered duodenal ulcer rebleeding. Eight ES patients suffered at least one episode of gastrointestinal bleeding: 4 from varices and 4 from esophageal ulcerations. Eight ES patients developed transitory dysphagia. Long-term follow-up was completed in all patients except for 5 cases (2 DSRS and 3 ES patients). Five-year survival rates for shunt (73%) and ES (56%) groups were statistically different: in this follow-up period and in subsequent follow-ups this difference decreased and ceased to be of statistical relevance. The primary cause of death

became hepatocellular carcinoma (HCC). Four DSRS patients rebled due to duodenal ulcer, while eleven ES patients had recurrent bleeding from esophago-gastric sources (seven from varices, three from hypertensive gastropathy, one from esophageal ulcerations) and two from unknown sources. Nine DSRS and 2 ES patients developed a chronic encephalopathy; 13 DSRS and 5 ES patients suffered at least one episode of acute encephalopathy. Five ES patients had esophageal stenoses, which were successfully dilated.

CONCLUSION: In a subgroup of patients with good liver function, DSRS with a correct portal-azygos disconnection more effectively prevents variceal rebleeding than ES. However, this positive effect did not influence the long-term survival because other factors (e.g. HCC) were more important in deciding the fate of the cirrhotic patients with portal hypertension.

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Key words: Esophageal varices; Portal hypertension; Liver cirrhosis; Shunt surgery; Endoscopic sclerotherapy

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INTRODUCTION

The selective distal spleno-renal shunt (DSRS) proposed by Warren^[1] in 1967 has been considered to be the best procedure available for surgical decompression of patients with portal hypertension^[2-4]. DSRS has been compared with sclerotherapy in four trials^[5-8]. Meta-analysis of these studies^[9] showed that DSRS significantly reduces the incidence of rebleeding and only slightly increases the occurrence of chronic encephalopathy, but does not improve survival. We began a prospective, randomized clinical trial to study the prevention of recurrent variceal bleeding in cirrhotic patients during long-term follow-up in 1984^[8]. We continued recruitment until 1991 and

continued to study the patients in the following years. In this interval of time, new procedures and new randomized trials were carried out, but as regards to survival, no treatment seems to be superior to another in patients with good or moderate liver function^[10]. As regards to other surgical options, use of a small-diameter prosthetic H-graft portacaval shunt has been compared with the transjugular intra-hepatic porto-systemic shunt (TIPSS), showing a favorable trend in survival in the surgical group^[11]. On the other hand, TIPSS has been compared with endoscopic sclerotherapy (ES) in 11 randomized trials and TIPSS seems, as confirmed by two meta-analyses^[12,13], to reduce rebleeding, but significantly increases encephalopathy without differences in survival. Furthermore, a number of trials comparing endoscopic band ligation with ES have been performed in the past few years and a meta-analysis of these studies^[14] showed that it is more effective than ES in preventing rebleeding, with no differences in survival.

Based on these analyses, it seems reasonable to choose as first-line therapy (i.e. beta-blockers, endoscopic band ligation, *etc.*) the least aggressive therapy^[15]. TIPSS or shunt surgery can be performed as a salvage treatment in patients who continue to rebleed and maintain good liver function. The purpose of this report is to describe the follow-up of patients in this study, which now extends beyond 20 years for surviving patients. Long-term follow-up presents the natural history of a subgroup of patients with portal hypertension in whom an active treatment (DSRS or ES) reduced the risk of rebleeding without causing irreversible damage to liver function.

MATERIALS AND METHODS

Patients

The study population comprised 80 patients with cirrhosis and portal hypertension referred to our department from October 1984 to March 1991. These patients were drawn from a pool of 282 patients who underwent either elective surgery or endoscopic sclerotherapy because of portal hypertension during this period of time.

Criteria for inclusion in the study were as follows: (1) liver cirrhosis confirmed by biopsy in all patients; (2) endoscopic documentation of variceal hemorrhage (actively bleeding varix or non-bleeding varices without other lesions) requiring at least one unit of blood transfusion; (3) arrest of acute variceal hemorrhage either spontaneously or by use of intravenous vasopressin and/or somatostatin and/or balloon tamponade and/or haemostatic sessions of ES; (4) less than 70 years old; (5) good or moderate liver function (Child's A and B class)^[16]; (6) patency of the splanchnic venous system and hepatopetal portal flow (according to Nordlinger's classification)^[17]; (7) eligible for either shunt or ES; (8) absence of life-threatening diseases (e.g. tumors); (9) willing to return for regular follow-up. Patients bleeding from gastric varices were excluded. Figure 1 shows the reasons for the exclusion of 202 patients.

Randomization for assignment into groups was carried out when the patient was stabilized, which occurred no more than 24 h before treatment. Patients were assigned to one of the 2 groups according to a random numbers table.

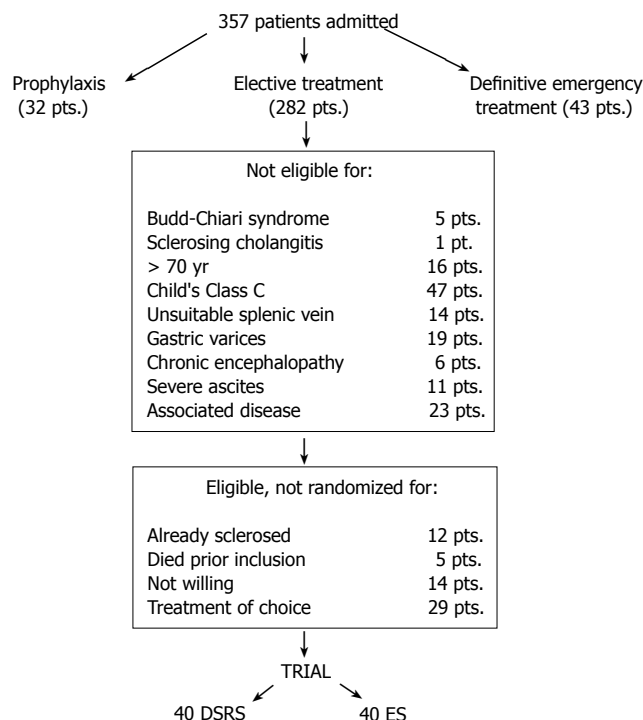


Figure 1 Reasons for exclusion. pts: patients.

Informed written consent was obtained from all patients prior to their inclusion in the study. No patient refused the assigned treatment.

Variceal rebleeding within 2 years of first treatment was considered as the primary measure of patient outcome. The sample size needed to show a decrease in variceal rebleeding from 43% to 7% is about 20 patients for each group, applying standard power (90%), type I error ($P < 0.05$) and a two-tailed t -test^[18].

Preoperative evaluation

A complete medical history was obtained for each patient, and particular notice was taken of previous episodes of gastrointestinal bleeding and evidence of either primary or post hemorrhagic hepatic failure (jaundice, ascites or edema). Routine laboratory tests were performed to evaluate liver function (Table 1). Overall assessment of the severity of liver disease was graded according to the Child-Pugh classification system^[16] and a personal hepatic score was obtained as previously described^[4,19]. Serum alpha-fetoprotein assessment and ultrasonography were routinely performed in order to screen for the presence of hepatocellular carcinoma. The presence of esophageal varices was assessed through endoscopic examination. Criteria used for classifying the endoscopic findings were based on the General Rules for Recording Endoscopic Findings on Esophageal Varices compiled by the Japanese Research Society for Portal Hypertension^[20].

Cerebral function was assessed through a complete neurological examination, taking into account mental state, asterixis, electroencephalographic findings (EEG), the trail making test^[21] and the "Cancelling A's" test^[22]. Our grading of hepatic encephalopathy (HE) has been previously described^[4,8,23]. We called HE "acute" if it was

precipitated by gastrointestinal bleeding, heavy drinking, pharmacological or dietary imbalances, of brief duration and easily controlled with elimination of the precipitating cause. We called HE “chronic” if it was spontaneous, of long duration and more difficult to manage. Naturally, preoperative HE was excluded in all patients.

A visceral angiogram was obtained by selective catheterization of the celiac axis and superior mesenteric artery. The degree of hepatic perfusion was evaluated according to Nordlinger's criteria^[17]. The rate of contrast infusion (sodium and meglumine ioxaglate, Hexabrix 320-Byk Gulden, Milan, Italy) infusion was 6 mL/s × 10 s.

Operative management

All shunt procedures were performed by a single experienced surgical team^[24]. DSRS was constructed according to the technique described by Warren^[25] (20 cases). In 18 cases a spleno-pancreatic disconnection^[26] was performed as a technical addition to DSRS, offering the optimum surgical therapy for each patient. In 2 patients a total shunt was performed for technical reasons; they were included in the statistical analysis on an intention-to-treat basis.

Endoscopic variceal sclerosis was conducted by 2 endoscopists with extensive experience in the field^[27]. All patients were given 5-10 mg diazepam premedication. ES was performed using an Olympus GIF IT flexible endoscope or an electronic Welch Allyn videoendoscope. At each session 10 to 50 mL of polidocanol (5-10 g/L) (Athoxysclerol, Creusller) and 5 g/L methylene blue were injected using a flexible injection needle in the area 5-7 cm above the esophago-gastric junction. Methylene blue allowed visual confirmation of intravariceal and paravariceal injections.

Postoperative evaluation

In the evaluation of hospital mortality and early complications, we defined the first 30 d after the initial treatment as the postoperative period. In the ES group, the events that occurred during the interval between the first session and eradication were also recorded and evaluated.

In the postoperative period, esophageal endoscopy was performed on each patient. A visceral angiography was performed on the 10th average postoperative day only in patients having DSRS. Shunt patency was verified in the venous phase of angiograms in the DSRS group. In the ES group, the number of further sessions depended on the findings obtained at endoscopy performed one week after the first sclerosis session. As soon as the eradication was achieved, the patient was included in the follow-up program. The remaining ES patients underwent further sclerosis sessions if they were free of complications, such as mucosal ulcerations, symptomatic stricture, severe esophagitis, fever and pneumonia. In the presence of complications, an upper endoscopy was performed at intervals of seven to ten days and further ES sessions were considered only when complications were resolved.

During the follow-up period, DSRS patients were checked at 1, 3 and 6 mo after discharge and then at least twice yearly, on an outpatient basis. Follow-up endoscopy was scheduled 4-6 wk after the last session and then at

Table 1 Characteristics of patients enrolled in the RCT

Characteristic	DSRS (n = 40)	ES (n = 40)	P
Age (yr)	49.4 ± 9.9	53.8 ± 8.4	0.027
Sex: M/F	27/13	33/7	0.121
Etiology: alcoholic/ non alcoholic	14/26	26/14	0.0073
Child's class: A/B	19/21	11/29	0.053
Prothrombin time: > 80/80-50/< 50	5%/82%/13%	13%/62%/25%	0.058
Bilirubin (μmol/L): < 20.5/20.5-51.3/> 51.3	62%/38%/0%	41%/46%/13%	0.0269
Albumin (g/L): > 30/30-25/< 25	95%/5%/0%	83%/17%/0%	0.077
Portal perfusion: degree I / II / III	45%/45%/10%	42%/27%/31%	0.09
Previous bleeding (n)	2.3 ± 1.5	1.6 ± 0.09	0.022
Blood unit (n)	5.5 ± 5	4.3 ± 4.2	0.25
Interval between bleeding and treatments (mo)	3.5 ± 2.5	2.8 ± 3.2	0.32
Previous encephalopathy episodes (n)	10	7	0.41
Easily controlled preoperative ascites	5	11	0.09

6-mo intervals, unless recurrent hemorrhage occurred.

At each visit, liver function was evaluated following a complete medical examination and laboratory tests. Assessment of the neurological status was performed using the above-mentioned criteria. An EEG was performed at least once a year. A return to drinking was ascertained based on patients' statements, our own assessment and information from relatives. Continued drinking was defined as daily consumption in excess of 1 liter wine and/or spirits. All patients were on a 10-meq sodium and protein-balanced diet (1 g protein/kg body wt) and undergoing lactulose prophylactic treatment: the initial dose was 60 g/d in 3 separate doses and adjusted thereafter to induce at least 1 bowel movement per day.

Definitions

Eradication was defined as the absence of varices or the presence of F1 white varices.

Rebleeding was defined as hemorrhage due to esophago-gastric varices and/or congestive gastropathy, requiring at least 1 unit blood transfusion and was designated as being from varices if this was supported by endoscopic findings. The treatment of choice for variceal rebleeding was emergency sclerotherapy. Chronic rebleeding from congestive gastropathy was treated with beta-blocking therapy^[28]. Rebleeding due to peptic ulcer was recorded separately. The risk of hospitalization for HE was defined by taking the number of late hospital admissions due to episodes of HE, and dividing it by the total number of patients evaluated in the follow-up period.

Data management and statistical analysis

Initial and subsequent data for the patients were collected on a dedicated spreadsheet (Excel, Microsoft Corp., Delaware, USA) for personal computer input (Macintosh G4, Apple Computer Inc.) and subsequent analysis

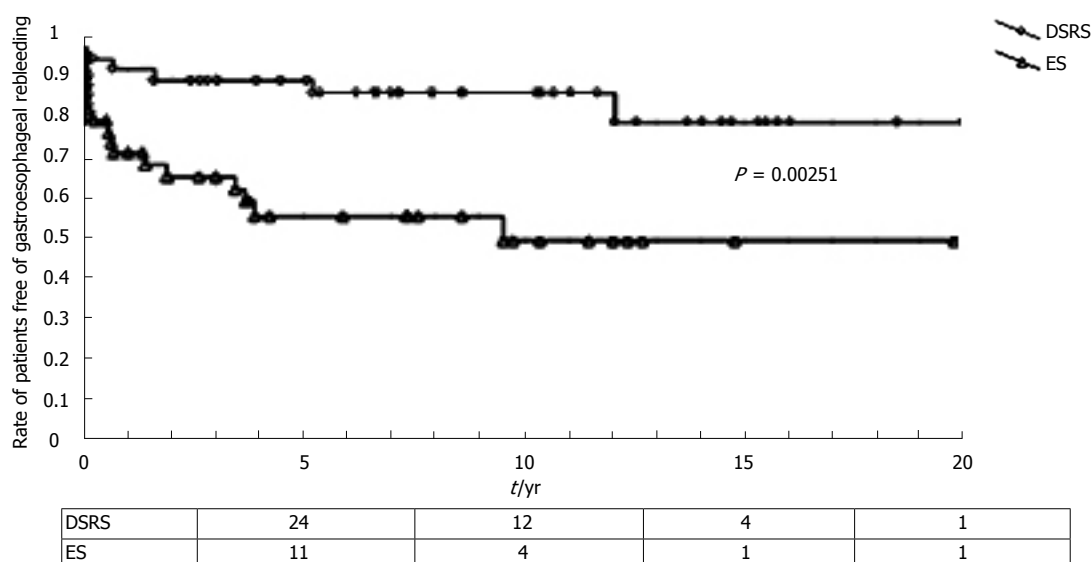


Figure 2 Actuarial curves of patients free from gastroesophageal rebleeding after distal spleno-renal shunt (DSRS) and endoscopic sclerotherapy (ES). $P < 0.05$ vs ES using the log-rank test.

(Statistica-Mac, Statsoft, Tulsa OK, USA). Survival and therapy failures were analyzed by the Kaplan-Meier method and were compared by Breslow and log-rank test^[29]. Comparison between groups was made by Chi-square test for proportions and Student's *t*-test for the means. In all patients, some preoperative variables were recorded and their influence upon survival in each treatment group was assessed by means of univariate analysis and Cox's proportional hazards regression model^[30]. The association of each parameter with survival was univariately estimated by comparing actuarial curves (Kaplan-Meier product-limit method and log-rank test) after categorization of the continuous variables in a multivariate setting^[31]. Only those parameters showing a statistical value of $P < 0.2$ were included in the multivariate analysis. The results of the univariate analysis helped to substantially reduce the number of prognostic factors. For each parameter analyzed in the multivariate analysis, the regression coefficient (beta), the *T*-values (Hazard ratio) and the 95% confidence intervals (CI) are given.

RESULTS

In the ES group, varices were completely eradicated in 36 patients (90%): 1 patient died before eradication and 3 patients abandoned the sclerotherapy program. The number of injection sessions was 3.7 ± 1.4 occurring over 5.1 ± 3.4 mo and the mean amount of polydocanol required for eradication was 84.7 ± 38.7 mL.

During the postoperative period, no DSRS patient died, while 1 ES patient died of uncontrolled hemorrhage after the first sclerosis session. One DSRS patient had mild recurrent variceal hemorrhage controlled by conservative therapy, despite an angiographically patent shunt and another patient rebled due to duodenal ulcer. Eight ES patients suffered at least one episode of gastrointestinal bleeding. Four were from varices requiring emergency variceal sclerotherapy and 4 from esophageal ulcerations, which were managed conservatively. No patient had episodes of hepatic encephalopathy during the immediate postoperative period. Eight ES patients developed

transitory dysphagia due to esophageal ulcerations. One ES patient suffered pleural effusion.

The mean follow-up in the DSRS group was 109 ± 58 (median 99.5) mo; ranging from 3 to 240 mo and the mean follow-up in the ES group was 87 ± 61 (median 71) mo; ranging from 1 to 237 mo. Long-term follow-up was complete in all patients, except for 5 cases (2 DSRS and 3 ES patients). No DSRS patient had shunt thrombosis, while 3 ES patients changed treatment: in 1 case a liver transplantation was successfully performed for liver failure and in 2 other cases a porto-caval H-graft shunting was performed for recurrent digestive bleeding. In the ES group, varices reformed in 14 of 36 eradicated patients (39%) after 20.4 ± 16.4 mo. They were successfully reeradicated. No DSRS patient had variceal rebleeding (four DSRS patients had duodenal ulcer rebleeding), while 7 ES patients had recurrent hemorrhage from varices, 3 from hypertensive gastropathy, 1 from esophageal ulcerations and 2 from unknown sources. The global percentage of patients who rebled was 10% and 33%, respectively ($P = 0.0194$). Figure 2 shows the actuarial curves of patients at risk of rebleeding. This risk was significantly higher in the ES group than in the DSRS group ($P = 0.00251$), above all for the first four years of follow-up. In the subsequent intervals of time, the two curves showed similar behavior.

There was no difference between patients with DSRS and ES in terms of preoperative results for the Trail making test, canceling A's test and EEG (Table 2). Postoperatively, the Trail making test showed no modifications in either group, while the canceling A's test showed an improved performance in both groups (probably due to a better comprehension of the test). EEG was unchanged after ES but worsened after DSRS. Thirteen DSRS (32.5%) and 5 ES patients (13%) suffered at least one episode of acute encephalopathy ($P = 0.0189$): three (2 DSRS and 1 ES) due to pharmacologic imbalance, three (2 DSRS and 1 ES) due to heavy drinking, three (1 DSRS and 2 ES) due to liver failure, seven (6 DSRS and 1 ES) due to constipation, one DSRS patient due to dietary abuse and one DSRS patient due to severe hyperglycemia. HE disappeared in all patients after medical therapy and dietary control in

Table 2 Comparison of the results of TMT, cancelling A's test and EEG. A's test refers to the number of A's the patients has omitted to cancel in the test

	Preoperative	Long-term follow-up	P
TMT (s)			
DSRS	55.3 ± 24.9	55.4 ± 27.9	NS
ES	60.1 ± 19.7	66.1 ± 40.1	NS
A's test			
DSRS	4.9 ± 3.8	2.6 ± 2.5	< 0.05
ES	5.5 ± 4.3	3.7 ± 3.4	< 0.05
EEG (% abnormal)			
DSRS	20%	36%	< 0.05
ES	16%	21%	NS

NS: Not significant.

Table 3 Causes of death of patients submitted to DSRS (*n* = 26) or ES (*n* = 31)

Causes of death	DSRS	ES
Digestive bleeding	1 (4%)	5 (16%)
Liver failure	6 (23%)	11 (36%)
Hepatocellular carcinoma	13 (50%)	10 (32%)
Other causes	6 (23%)	5 (16%)

all patients. Nine DSRS (22.5%) and 2 ES patients (5%) developed a mild chronic encephalopathy: the difference in the incidence of chronic HE between DSRS and ES was statistically significant ($P = 0.0263$). However, the risk of hospitalization for HE was not different: 0.38 ± 0.4 and 0.28 ± 0.3 after DSRS and ES, respectively ($P = \text{NS}$). In summary, HE was more frequent in the DSRS group than in the ES group, but not more severe. Seven DSRS patients (17.5%) and 9 ES patients (23%) returned to drinking ($P = \text{NS}$). Five ES patients had esophageal stenosis that was successfully dilated. Ascites developed in 11 DSRS patients (27.5%) and in 19 ES patients (49%; $P = 0.0521$).

Long-term survival rates for shunt and ES groups were not statistically different according the log-rank test (Figure 3); however, a statistical difference was found by the Breslow test which better reflects the initial results. Causes of death are shown in Table 3: hepatocellular carcinoma became the primary cause of death with an increase in follow-up time (Figure 3). In the first five-year follow-up, the primary cause of death was liver failure (38%). No differences were found with respect to the etiology of liver cirrhosis: HCC developed in 35% of patients with non-alcoholic cirrhosis and in 32.5% of patients with alcoholic cirrhosis. Table 4 shows the results (actuarial survival curves and log-rank test) of the univariate analysis of some variables calculated at different follow-up intervals. By multivariate analysis according to Cox's model, age over or below 53 years old was the only independent predictor of global survival rate.

DISCUSSION

The design of a randomized, controlled trial (RCT)

Table 4 Results (actuarial survival curves and log-rank test) of the univariate analysis of some variables calculated during different interval of follow-up

Variables	P (at 5 yr)	P (at 10 yr)	P (total follow-up)
Type of treatment (DSRS vs ES)	0.0385	0.1415	0.1168
Sex	0.2186	0.3374	0.1943
Child's score (A vs B)	0.0358	0.2533	0.1594
Etiology of cirrhosis (non-alcohol vs alcohol)	0.2891	0.0749	0.0438
Portal perfusion	0.2671	0.3579	0.3158
Preop. Ascites	0.3339	0.9022	0.9254
Preop. encephalopathy	0.6544	0.9857	0.8732
Preop. Varices	0.6513	0.4552	0.3858
No. of previous bleeding	0.1724	0.2739	0.1308
Preop. Bilirubin	0.2944	0.3358	0.0604
Preop. GOT	0.4504	0.2659	0.2553
Preop. Prothrombin time	0.2835	0.5629	0.4815
Preop. Albumin	0.7440	0.2133	0.1823
Age (< 53 yr)	0.1419	0.0431	0.0438
Postop. Rebleeding	0.1715	0.1168	0.0819

Preop: Preoperative; Postop: Postoperative.

comparing surgical treatment with a conservative therapy like ES, is often a complicated process requiring a choice of end-point, patient selection criteria, treatment schedules and methods of patient evaluation. In addition to our study^[8], three other RCTs comparing DSRS to ES^[5-7] have been published. They took different approaches to study these issues. It is true that survival is the primary end-point in prospective studies. In 2 studies^[5,6] the definition of major end-point was not specified (survival, prevention of recurrent bleeding and maintenance of hepatic function) and no statistical method for determining trial size was used. The study of Terés^[7] was designed to observe an increase in survival. However, the expected increase in survival appears to be too optimistic. On the basis of the available data^[2,24,32-35], the sample requirements needed to show a realistic 10% increase in survival at 5 years is about 470 patients per group, applying standard power (80%), type I error ($P < 0.05$) and a two-tailed *t*-test^[36].

Since gastro-esophageal rebleeding is considered the most life-threatening complication in ES, we wanted to verify whether DSRS was more effective in the prevention of rebleeding than ES and evaluate if this improvement affected survival. We limited the necessary recruitment to 20 patients per group, so the trial needed only one surgical team. This consideration is a general problem in surgical multicenter trials, as the surgeon's skill is a major factor in the surgical outcome, especially in the case of a technically complex operation such as DSRS^[37]. Our trial was undertaken after the team had acquired experience over 80 selective shunt procedures, to ensure a fairly good standardization of the surgical approach and intra- and post-operative treatment^[24]. We then decided to increase the number of patients to be recruited to 40 patients per group in order to either eliminate the difference observed for the preoperative variables or carry out a subgroup analysis. The results of these trials^[5-7] confirm

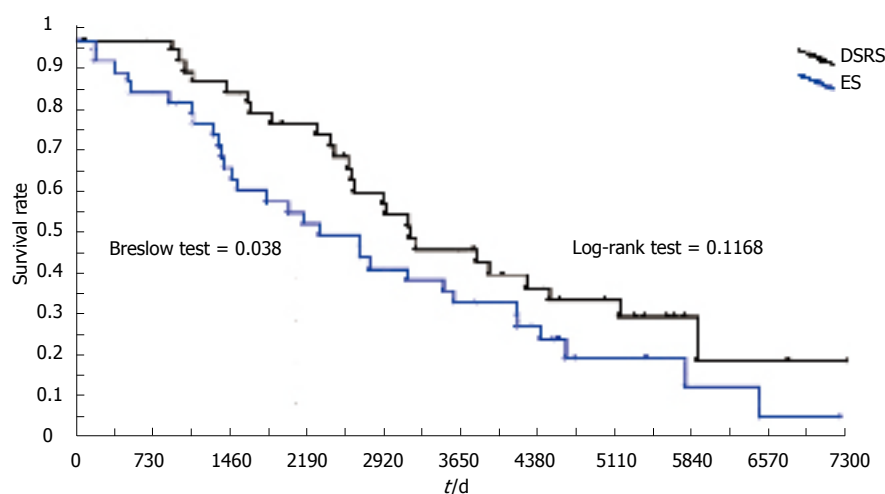


Figure 3 Actuarial survival curves for distal spleno-renal shunt (DSRS) and endoscopic sclerotherapy (ES). DSRS vs ES for the first 5 years (breslow test $P = 0.038$) while no differences were found in the final outcome (log rank test $P = 0.1168$)

DSRS	39	33	28	20	16	11	6	3	2	1
ES	33	25	20	16	12	8	4	2	1	1

previous results showing that DSRS is more effective in preventing variceal rebleeding than ES (Table 5). Either gastroduodenal lesions or esophageal varices can cause gastro-esophageal rebleeding after DSRS. The most common cause of variceal rebleeding is shunt thrombosis, but hemorrhage can also occur when the shunt is patent, because of renal vein hypertension^[38].

The problem of rebleeding in the ES group is complex and is connected with a number of factors, such as the number of patients with eradicated varices, the time interval required for eradication, the incidence of sclerosis-induced mucosal ulcerations of the esophageal wall, the variceal relapses after eradication and the incidence of hypertensive gastropathy^[39]. Our incidence of rebleeding (35%) is similar to that reported by Terés^[7] but lower compared to other studies^[5,6]. The relevance of the difference in techniques in explaining these differences is not clear^[40,41]. Different treatment intervals have been shown to affect the incidence of variceal rebleeding^[42]. Finally, it is a common experience that early rebleeding occurs frequently in Child's C patients^[5] and the number of Child's patients differed in all these RCTs.

The main complication in shunted patients was chronic HE that little affected ES patients. In the 4 RCTs the incidence of HE was higher in the DSRS group than in the ES group. This trend became significant in Terés's study^[7], probably due to the modified technique employed, which did not associate portal-azygos disconnection with the spleno-renal shunt. In fact, the entity of collaterals after DSRS seems to be an important factor influencing the incidence of HE^[43,44]. In Rikkers's^[6] and Warren's^[5] studies chronic HE was probably attributed to the hepatic failure, and that was due to the fact that both Child's C patients^[5,6] and total shunts^[6] were taken into consideration. In our preliminary study^[8], the low rate of chronic HE in the DSRS group could be due to the recruitment of patients with good liver function and a shorter follow-up period than in other studies. In fact, the lengthening of the follow-up led to an increased risk of developing HE, above all in patients who underwent surgical shunt, but the quality of life was only slightly affected, as confirmed

Table 5 2-yr survival and rebleeding rate in randomized controlled trials comparing DSRS with ES

	Warren ^[5]	Terés ^[7]	Rikkers ^[6]	Our data
2-yr survival rate after DSRS	59% ^a	71%	65%	95%
2-yr survival rate after ES	84% ^a	68%	61%	85%
Child's C patients	43%	0	33%	0
Interval between bleeding and treatment (d)	> 3-5	10-15	NR	901
Rebleeding rate after DSRS	3% ^a	14% ^a	19% ^a	2.5% ^a
Rebleeding rate after ES	53% ^a	37% ^a	57% ^a	35% ^a
Variceal eradication rate	NR	46%	63%	90%
Failure to salvage rebleeders	17%	33%	47%	14%
Shunt for ES failure	31%	6%	7%	5%
Global mortality rate due to rebleeding	3%	14%	27%	16%
Global mortality rate due to other causes	13%	16%	13%	17%

NR = Not reported; ^amean value; ^a $P < 0.05$ vs ES.

by the same incidence of hospitalization rate. In fact, the selective effect of DSRS prevented the onset of the severe form of chronic HE.

Two-year survival rate ranged from 59% to 95% in DSRS and from 61% to 90% in the ES group (Table 5). Where do these great differences stem from? The different incidence in these studies for two prognostic factors (Child's class C patients and the interval between hemorrhage and treatment) can explain these results^[45]. However, a more important factor is present in the ES group: the fate of the patient after variceal rebleeding (Table 5). The fact that some studies^[6,7] do not have available a good therapeutic option available in case of ES failure can influence these results. The better survival rate reported in Warren's

study^[5] seems to be due to the large number of ES patients who underwent shunt surgery (10 out of 36 patients) with low operative mortality (10%). This advantage was not shown in the other studies^[6,7].

The lack of statistical difference observed in our study was also confirmed by meta-analysis of the four RCTs: the overall risk of death following DSRS was only marginally lower in comparison to that following ES (0.78 odds ratio; 95% CI: 0.47-1.29). In this study, we found a statistically improved survival following DSRS with the Breslow test; it gives more emphasis to early deaths occurring in the ES group. When lengthening the follow-up period, the survival curves become more similar and the risk of rebleeding does not increase with increasing follow-up time, while the appearance of HCC heavily influenced long-term survival in both groups, becoming the primary cause of death. The Breslow test better reflects initial results while the log-rank test more accurately characterizes final outcome.

In contrast with a recent study^[46], our analysis did not find any significant differences regarding the development of HCC (44% in DSRS group *vs* 42% in ES group even though there was a higher proportion of patients' deaths due to HCC (50% of all the causes of death in the DSRS group *vs* 32% in the ES group). This finding should be interpreted cautiously since this trial was not specifically designed to evaluate the effect of DSRS or ES on the development of HCC. In any case, it is possible that the surgical access of the DSRS could promote an HCC diagnosis at a more advanced stage and prevent the use of specific therapies for HCC, such as radiofrequency (through a percutaneous or a laparoscopic approach) or surgical resections. Another study^[47] concluded that DSRS was not a problem preventing safe performance of therapies for the HCC that developed during the follow-up period. On the other hand, it has been shown that patients with esophageal variceal bleeding who underwent ES had a high risk of developing HCC with an adverse impact on survival^[48]. Further studies are necessary to evaluate the correlations between the therapies for portal hypertension and the appearance of HCC. Furthermore, in our analysis, no differences were found regarding the etiology of liver cirrhosis, although it is not possible to know how many patients had an HCV-related cirrhosis because the test has only been used in our Department since 1991. Hepatitis C disease has assumed increasing importance over the past years and undoubtedly contributed to cirrhosis and HCC in patients with cryptogenic or alcoholic liver disease before serologic markers became routinely available.

Multivariate analysis by Cox's model found that the only factor contributing independently to the estimation of prognosis was the age of patients at the time of inclusion in the study (Hazard risk: 2.339; coefficient beta: 0.849; T-value: 2.729). That age was a prognostic variable is not surprising, since it reflects a longer duration of follow-up.

Meanwhile, in 2006, what is the lesson provided by the analysis of such a long-term follow-up study? First of all, although it is clear that all patients who survive a variceal bleeding should be treated with beta-blockers or endoscopic band ligation, which appears to be better than ES in preventing rebleeding, it is also clear that all patients in whom bleeding cannot be controlled or who continue to

rebleed can be treated with salvage TIPSS or shunt surgery, even though some studies seem to show the superiority of surgical over angiographic shunt in good-risk patients^[11,49]. Secondly, although the number of patients who have uncontrolled rebleeding following beta-blockers or endoscopic band ligation are very few, it is important that there be centers with qualified surgeons who can offer DSRS to selected patients to guarantee long-term survival comparable to that obtained by liver transplantation. Finally, it is important to underline that, in the evaluation of chronic disease with multiple complications such as liver cirrhosis, the time factor should be considered and the effect of each therapy should also be analyzed over a long-term follow-up period. This work confirms that it is possible to perform a shunt procedure without impairing liver function or the prognosis of the cirrhotic patient.

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Comparison of a new aspiration needle device and the Quick-Core biopsy needle for transjugular liver biopsy

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Abstract

AIM: To evaluate sample adequacy, safety, and needle passes of a new biopsy needle device compared to the Quick-Core biopsy needle for transjugular liver biopsy in patients affected by liver disease.

METHODS: Thirty consecutive liver-disease patients who had major coagulation abnormalities and/or relevant ascites underwent transjugular liver biopsy using either a new needle device (18 patients) or the Quick-Core biopsy needle (12 patients). The length of the specimens was measured before fixation. A pathologist reviewed the histological slides for sample adequacy and pathologic diagnoses. The two methods' specimen adequacy and complication rates were assessed.

RESULTS: Liver biopsies were technically successful in all 30 (100%) patients, with diagnostic histological core specimens obtained in 30 of 30 (100%) patients, for an overall success rate of 100%. With the new device, 18 specimens were obtained, with an average of 1.1 passes per patient. Using the Quick-Core biopsy needle, 12 specimens were obtained, with an average of 1.8 passes per patient. Specimen length was significantly longer with the new needle device than with the Quick-Core biopsy needle ($P < 0.05$). The biopsy tissue was not fragmented in any of the specimens with the new aspiration needle device, but tissue was fragmented in 3 of 12 (25.0%) specimens obtained using the Quick-Core biopsy needle. Complications included cardiac arrhythmia in 3 (10.0%) patients, and transient abdominal pain in 4 (13.3%) patients. There were no cases of subcapsular hematoma, hemoperitoneum, or sepsis, and there was no death secondary to the procedure. In particular, no early or delayed major procedure-related complications were observed in any patient.

CONCLUSION: Transjugular liver biopsy is a safe and effective procedure, and there was significant difference in the adequacy of the specimens obtained using the new needle device compared to the Quick-Core biopsy needle. Using the new biopsy needle device, the specimens showed no tissue fragmentation and no increment in major procedure-related complications was observed.

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Key words: Liver disease; Biopsy-interventional procedures; Transjugular biopsy

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INTRODUCTION

To diagnose the cause and stage of liver disease, transjugular liver biopsy is an effective way to obtain a liver specimen in patients with a contraindication to percutaneous biopsy, such as coagulopathy or ascites^[1-3]. Several different kinds of biopsy needles have been designed, including the modified Ross needle (Cook, Bloomington, Indiana) placed through a 9-F curved sheath^[4], and a side-cutting needle, such as the Quick-Core needle (Cook Medical, Bloomington, IN, USA) placed through a 7-F curved sheath with a guiding metal cannula inside the sheath^[5]. A widely used, single-use biopsy needle, the Quick-Core system uses an 18-, 19-, or 20-gauge cutting needle with an automated spring-fired mechanism to obtain tissue. The popularity of the Quick-Core system is its relative ease of use and high diagnostic yield. The success rate for transjugular liver biopsy is high, but sometimes tissue samples inadequate to secure a diagnosis are obtained by this device. The present report describes the results obtained by a new biopsy device of our own design for transjugular liver biopsy.

In our hospital, we use two types of needle: a new aspiration needle device of our own design (supplied by Hakko Co Ltd, Nagano, Japan) and the Quick-Core biopsy

needle (Cook Medical). To our knowledge, there have been only a few reports comparing the safety and adequacy of the different types of needles. The purpose of this study was to evaluate and compare the safety, complications, and tissue sample adequacy, including the diagnostic yield, of transjugular liver biopsy using the two different needles.

MATERIALS AND METHODS

Between April 2002 and April 2006, 30 patients had a transjugular liver biopsy at Saiseikai Niigata Second Hospital. There were 15 men and 15 women, aged 22-75 years, with an average age of 46.57 years. All cases had a contraindication for percutaneous liver biopsy due to coagulation abnormalities and/or massive ascites. Coagulation abnormalities were defined as an INR of 1.4 or greater, and a platelet count less than 30 000/cm³. After sedation and local anesthesia, a transjugular liver biopsy was performed in 18 patients using an 18-gauge new aspiration needle device (Figure 1A), and in 12 patients using the 18-gauge Quick-Core Biopsy Needle (Figure 1B) System. With the new device, histological core liver specimens were obtained by manually aspirating through the inner cannula. All patients gave their informed consent after being fully informed of the nature of the study. The study was approved by the Ethical Committee of our hospital and was conducted in accordance with the principles of the Declaration of Helsinki. Patients were randomly allocated using a computer generated randomization code to undergo transjugular liver biopsy with the new device or with the automated Quick-Core device. The randomization results were securely kept in sealed opaque envelopes.

Technique

A 9-F, 45-cm vascular sheath (Cook Medical), was placed over a 0.035-inch guide wire into the right internal jugular vein according to the Seldinger technique, and then a 7-F multipurpose catheter was advanced under fluoroscopic control into the right, middle, or left hepatic vein. Subsequently, contrast venography was done to determine the actual position of the catheter before biopsy. With the new device, the aspiration biopsy needle was advanced within the catheter to its tip, and then the specimen was obtained by aspiration while further pushing it into the parenchyma in an antero-inferior direction. With the 18-gauge, 60-cm-long, side-cutting automated biopsy device (Quick-Core needle) the needle was positioned in the same direction, and one or several passes were done to obtain an adequate sample. Fluoroscopy and ECG monitoring were used throughout the procedure in both groups.

After the biopsy procedure, contrast medium was injected through the catheter to rule out capsular perforation. The retrieved specimens were mounted longitudinally on dry paper, and their lengths were measured before formalin fixation. An adequate amount of liver tissue was obtained and placed in formalin for pathologic evaluation. The samples were considered fragmented if the largest core sample was less than 6 mm in length. The procedure was considered to be

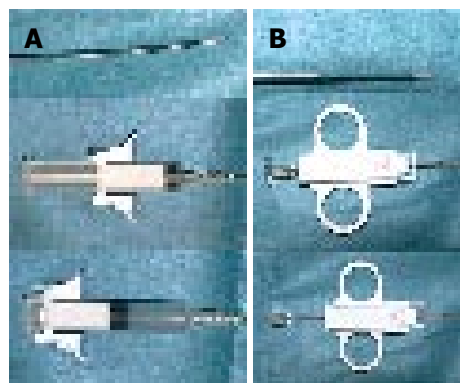


Figure 1 A: Top: Edge of new needle device; Middle: The plunger of the new needle device in the puncture position; Bottom: The plunger of the new needle device in the aspiration position, obtained by pulling back until a firm click is felt; B: Top: A 20-mm specimen notch of the Quick-Core biopsy needle; Middle: The plunger of the Quick-Core biopsy needle; Bottom: The Quick-Core biopsy needle is prepared by pulling back on the plunger until a firm click is felt, which indicates that the needle spring is locked in the ready position.

Table 1 Indications for transjugular approach to liver biopsy

(1) Increased risk of liver bleeding: 16 cases (53.3%) Severe coagulation disorders (14 cases) including: -Thrombocytopenia: 1 cases (due to hypersplenism or hematological disorders) -Fulminant hepatitis (Severe acute hepatitis): 13 cases Miscellaneous disorders (2 cases) including: -Hematological diseases (DIC): 2 cases
(2) Measurement of hepatic pressure gradient: 4 cases (13.3%)
(3) Massive ascites: 5 cases (16.6%)
(4) Miscellaneous: suspicion of amyloidosis or peliosis, obesity, failure of percutaneous liver biopsy: 5 cases (16.6%)

technically successful if a liver tissue sample was obtained. If the pathologist was able to determine the histological diagnosis based on the sample provided, the procedure was considered diagnostic. One experienced hepatobiliary pathologist was not informed about the technique. She evaluated the histological slides to obtain a pathologic diagnosis and to determine the adequacy of the submitted specimens. Complications during the procedure were recorded, and patients' clinical records were reviewed for any delayed complications. The Mann-Whitney *U*-test was used to statistically analyze any differences between the two groups.

RESULTS

Indication

Table 1 presents the indications for transjugular biopsy in our hospital (Table 1). In 70.0% of the patients in this study, coagulation disorders, thrombocytopenia, or ascites were the indications for transjugular biopsy.

Clinical Features

The clinical features of the patients included in the study and the indications for biopsy were similar between the two groups (Table 2).

Table 2 Clinical features of patients

Clinical feature	New device (<i>n</i> = 18)	Quick-core biopsy needle (<i>n</i> = 12)
Sex (M/F)	9/9	6/6
Mean age \pm SD (range) (yr)	46.6 \pm 15.4 (22-72)	49.4 \pm 16.3 (27-75)
Indications		
Increased risk of bleeding:	9	7
Measurement of hepatic pressure gradient:	3	1
Massive ascites:	3	2
Miscellaneous:	3	2

Table 3 Final diagnosis of successful biopsies (*n* = 30)

(1) Liver cirrhosis: 9 cases (30.0%)
Alcohol: 5 cases AIH: 3 cases IPH: 1 case
(2) Viral hepatitis: 14 cases (46.6%)
Fulminant: Massive necrosis: 4 cases, Submassive necrosis: 9 cases, Chronic: 1 case
(3) Unknown Origin: 2 cases (6.6%)
Focal necrosis: 2 cases
(4) Tumoral diseases: 2 cases (6.6%)
Metastasis: 1 case, Lymphoma: 1 case
(5) Others: 3 cases (9.9%)
Intrahepatic cholestasis: 1 case, NASH s/o: 1 case, Amyloidosis s/o: 1 case

Histological Findings (Table 3)

The histological diagnoses included: cirrhosis (9 patients, 30.0%), viral hepatitis (14 patients, 46.6%), focal necrosis (2 patients, 6.6%), tumoral diseases (2 patients, 6.6%), and others (3 patients, 9.9%).

Biopsy Specimens obtained from successful attempts (Table 4)

Transjugular biopsy was technically successful in 30 of 30 (100%) cases. A total of 30 specimens (18 for the new needle device and 12 for the Quick-Core biopsy needle) were obtained, with an average of 1.4 needle passes per patient (1.1 for the new needle device and 1.8 for the Quick-Core biopsy needle). However, the tissue was fragmented in 3 (25%) of 12 specimens obtained using the Quick-Core biopsy needle. In contrast, there was no tissue fragmentation in any of the 18 specimens obtained using the new aspiration needle device. Furthermore, all 12 specimens obtained using the Quick-Core biopsy needle were shorter than 10 mm, probably because of insufficient parenchymal puncture. Although sample fragmentation was present in 3 (25%) cases, all 12 procedures were diagnostic (100%).

The lengths of the specimens obtained using the Quick-Core biopsy needle were significantly shorter (3-10 mm, mean = 6.8 mm) than the lengths of the specimens obtained using the new needle device (10-21 mm, mean = 15.3 mm, $P < 0.05$). All of the specimens obtained using the new biopsy needle device were adequate for making a diagnosis (100%). Transjugular liver biopsy with the new aspiration device was more effective than with

Table 4 Biopsy specimens obtained in successful attempts

	New device (<i>n</i> = 18)	Quick-core biopsy needle (<i>n</i> = 12)
Tissue Length (mm)	15.3 \pm 5.1 (10-21)	6.8 \pm 2.1 (3-10)
Number of portal triads	6.5 \pm 1.2	3.5 \pm 0.6
Number of fragments	0/18 (0%)	3/12 (25%)
Number of passes	1.1 \pm 0.2	1.8 \pm 0.8
Minutes (min)	28.5 \pm 7.9	45.1 \pm 9.9

Table 5 Complications of transjugular liver biopsy

	New device (<i>n</i> = 18)	Quick-core biopsy needle (<i>n</i> = 12)
Cardiac arrhythmia		
(Supraventricular tachycardia)	1	2
Transient fever	0	1
Transient abdominal pain	1	3

the Quick-Core biopsy needle with respect to obtaining an accurate diagnosis. The mean procedure time was statically significant shorter using the new needle device (28.5 \pm 7.9 min) than with the Quick-Core biopsy needle (45.1 \pm 9.9 min).

Complications (Table 5)

There were no early or late complications, such as capsular perforation, hemoperitoneum, or puncture site hematoma with either device.

Three minor complications were reported in 8 (26.6%) patients; there were no major complications. Three patients developed supraventricular tachycardia during the procedure, which was believed to have been a pre-existing condition. The patients' supraventricular tachycardia responded to medical therapy with conversion to normal sinus rhythm within 24 h. Other minor complications included transient abdominal pain ($n = 4$) and transient fever ($n = 1$) that spontaneously resolved before the procedure was completed. The complication rate with the new device was significantly lower than with the Quick-Core needle.

DISCUSSION

Percutaneous liver biopsy allows one to obtain adequate tissue samples with minimal injury to the liver. However, the procedure is contraindicated in patients with an increased risk of hemorrhage, such as those with marked coagulopathy, thrombocytopenia, or ascites^[6,7]. In 1964, Dotter reported successfully biopsying the livers of several dogs using a transvenous catheter^[8]. Subsequently, Hanafee and Weiner first described the technique of transjugular catheterization of the hepatic veins in human subjects^[9]. Three years later, the first human transjugular liver biopsies were performed in a few patients by the same authors^[10].

In 1973, Rosch and colleagues reported the first major clinical series of transjugular liver biopsy with a description of 44 biopsies^[11].

Today, transjugular liver biopsy is an established alter-

native procedure for patients who have a contraindication for percutaneous biopsy. The factors that affect the quality of the specimen obtained are: type of needle, operator skill, and texture of the liver parenchyma. The selection of a needle for doing liver biopsy is based on needle safety, ease of manipulation, and reliability in retrieving specimens of an adequate length that are not fragmented. The new needle device and the Quick-Core biopsy needle use different mechanisms to obtain tissue samples. The new device uses an aspiration (suction) mechanism, while the Quick-Core device uses a swift side-cutting motion (Figure 1). The Quick-Core biopsy needle is part of a semi-automated 18-gauge Trucut needle biopsy gun device, composed of a cutting cannula and an inner stylet with a 20-mm specimen notch. Papatheodoridis *et al*^[12] found no difference in the adequacy of the specimens obtained by the Quick-Core system and the Colapinto needle, but they did not specify the core size.

We report our experience of 18 transjugular liver biopsy procedures using the 18-gauge new needle device system. The technical success rate of the biopsy was 100%. In our study, there were no cases of intraperitoneal or subcapsular bleeding; this may have been due to the use of routine venography after the biopsy passes.

DeHoyos *et al*^[13] reported a mean of 2.4 ± 1.0 passes, Papatheodoridis *et al*^[12] reported a mean of 1.8 ± 1.0 passes, Choh *et al*^[14] reported a mean of 2.8 passes, and Gonzalez-Tutor *et al*^[15] reported a mean of 3.1 passes. A larger number of needle passes contributes to a higher incidence of complications, most notably perforation of the liver capsule. In our study, 30 specimens (18 with the new needle device and 12 with the Quick-Core biopsy needle) were obtained, with an average of 1.4 needle passes per patient (1.1 for the new needle device and 1.8 for the Quick-Core biopsy needle). The number of passes was significantly lower with the new needle device than with the Quick-Core system.

The specimens obtained were significantly longer with the new needle device (10-21 mm, mean = 15.3 mm) than with the Quick-Core biopsy needle (3-10 mm, mean = 6.8 mm, $P < 0.05$).

The transjugular liver biopsy specimens obtained using the new needle device had a high diagnostic yield comparable to those obtained using the Quick-Core biopsy system. A critical disadvantage of the Quick-Core system needle is frequent tissue fragmentation. Previously, transjugular liver biopsy done using conventional aspiration techniques has been shown to yield insufficient tissue samples. It is known that a suction or aspiration biopsy of a cirrhotic liver is likely to result in tissue fragmentation. Using the Quick-Core biopsy needle, the tissue was fragmented in 3 (25%) of 12 specimens, while there was no tissue fragmentation in any of the 18 specimens obtained using the new aspiration needle device.

Therefore, the new needle device system would appear

to be superior to the Quick-Core system transseptal needle for obtaining pathologically adequate specimens. The length of all specimens obtained with the Quick-Core needle was less than 10 mm, due primarily to insufficient puncture of liver parenchyma. In our study, no procedure-related problems occurred with our new needle device system. There were no cases of intraperitoneal hemorrhage, even without the insertion of plugging material into the biopsy tract.

In conclusion, transjugular liver biopsy is a safe and effective procedure in high-risk liver-disease patients for whom percutaneous biopsy is contra-indicated. With the new biopsy needle device, larger specimens are usually obtained without tissue fragmentation. In summary, transjugular liver biopsy using the new needle device is safe and has a high diagnostic yield in patients with advanced liver disease.

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Lack of association between *UGT1A7*, *UGT1A9*, *ARP*, *SPINK1* and *CFTR* gene polymorphisms and pancreatic cancer in Italian patients

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Abstract

AIM: To investigate simultaneously *UGT1A7*, *UGT1A9*, *ARP*, *SPINK1* and *CFTR* genes to verify whether genetic polymorphisms predispose to the development of pancreatic cancer (PC).

METHODS: Genomic DNA of 61 pancreatic cancer patients and 105 healthy controls (HC) were analyzed. *UGT1A7* genotyping was determined by PCR-RFLP analysis. Specific PCR and sequencing were used to analyze genetic variants of *UGT1A9*, *ARP*, *SPINK1* and *CFTR* genes.

RESULTS: Four different alleles (*1: WT; *2: N129K and R131K; *3: N129K, R131K, and W208R; and *4: W208R) in *UGT1A7* and three different alleles (*1: WT; *4: Y242X; and *5: D256N) in *UGT1A9* were detected. All *UGT1A* polymorphisms were observed at similar frequency in PC patients and HC. Seven different alleles in *ARP* were found in PC patients and HC at similar frequency. The *SPINK1* mutations N34S and P55S occurred in five PC patients with a prevalence (4.1%) not significantly different from that observed (2.0%) in HC. The only *CFTR* Δ F508 mutation was recognized in three PC patients with a prevalence (4.9%) similar to HC.

CONCLUSION: *UGT1A7*, *UGT1A9*, *ARP*, *SPINK1* and *CFTR* gene polymorphisms are not associated with PC in Italian patients.

INTRODUCTION

Pancreatic cancer (PC) is still a major cause of morbidity and mortality worldwide. Despite extensive research, its etiology remains elusive. Recent data suggest that both genetic and environmental factors play a role in the development and progression of PC.

In 5%-10% of cases, PCs are inherited either as part of a known familial cancer syndrome or in association with familial forms of chronic pancreatitis^[1]; in contrast, in 90%-95% of PC cases, the relationship between possible genetic predisposition and environmental risk factors is less clear. Tobacco smoking is actually recognized as the only environmental risk factor in at least 30% of all sporadic PC^[2], while the role of alcohol and other xenobiotics is still controversial.

Polymorphisms in low penetrance genes involved in carcinogen metabolism and oxidative stress, such as *CYP1A1*, *CYP2D6*, *GSTM1*, *GSTT1*, *NAT1*, *UGT1A7* and *UGT1A9*, could increase susceptibility to PC through exposure to endogenous metabolites or environmental xenobiotics^[3]. Interestingly, among all these detoxifying activities, the *UGT1A7* and *UGT1A9* seem to be the only enzymes capable of tobacco-borne toxicant inactivation^[4-6]. Only few studies have investigated the polymorphism of *UGT1A7* and *UGT1A9* genes in PC patients with controversial findings^[7,8].

The known pancreatic cancer susceptibility genes include germline mutations of high penetrance tumor suppressor genes or oncogenes involved in cell cycle control and DNA repair such as *CDKN2A* (also known as *p16*), *p53*, *DPC4* (also known as *SMAD4*), *STK11*, *BRCA2*,

K-ras, *hMLH1* and arginine-rich protein (*ARP*). The effect of these gene mutations in inducing PC seems to be poorly influenced by environmental factors^[2]. In particular, *ARP* gene has been described as oncogene. Although the functional role of the encoded protein is still unknown, the gene has been found in all species and is highly conserved at the DNA and RNA level^[9]. The presence of multiple AGG repeats around codon 50 of the mRNA and the gene has been reported in a variety of solid tumors^[10,11]. Thus, variations in the ARP trinucleotide repeat region correspond to polymorphisms of the gene^[12].

The known familial chronic pancreatitis susceptibility genes include germline mutations of moderate penetrance genes, such as cationic trypsinogen (*PRSS1*)^[13], serine protease inhibitor Kazal type 1 (*SPINK1*)^[14-16] or cystic fibrosis transmembrane conductance regulator (*CFTR*)^[17] which determine hereditary pancreatitis (HP)^[13], idiopathic chronic pancreatitis (ICP)^[15] and cystic fibrosis (CF)^[17], respectively. These genes are involved in trypsinogen activation and chloride transport, cause long-standing chronic pancreatitis and, in combination with other environmental factors such as smoking, may induce PC. However, when the polymorphisms of these genes were investigated in patients with PC, data have been inconclusive^[18,19].

We hypothesize that patients with PC could have an asymptomatic chronic pancreatitis due to the interaction of environmental factors (smoking and other xenobiotics) and genetic variations of genes involved in chronic pancreatitis susceptibility or detoxifying activities (i.e. *UGT1A7*). Moreover is conceivable that these genes may interact with each other and with other known risk factors (i.e. smoking) thus increasing the risk for PC. A possible explanation of the inconclusive results of the previous studies could be the evaluation of polymorphisms of single gene. This study focused on the simultaneous investigation of the genetic variations in *UGT1A7*, *UGT1A9*, *ARP*, *SPINK1* and *CFTR* genes as potential risk factor for sporadic PC in an Italian population cohort. A possible gene-gene interaction and correlation with age at diagnosis and smoking habit was also evaluated.

MATERIALS AND METHODS

Subjects

We investigated the prevalence of *UGT1A7*, *UGT1A9*, *ARP*, *SPINK1* and *CFTR* gene polymorphisms in 61 consecutive unrelated patients with sporadic pancreatic adenocarcinoma (PC) (mean age: 63 ± 10 years; range: 44-81 years; male/female: 31/30) and in 105 healthy blood donors (HC) (mean age: 39 ± 9 years; range: 19-60 years). Information on histological diagnosis and tumor location were available for all patients with pancreatic cancer (Table 1). DNA from patients and controls was obtained from peripheral blood samples after written informed consent was given. The study was approved by the Ethics Committee of our hospital.

Genotyping of *UGT1A7* (MIM: 606432)

UGT1A7 exon 1 polymorphic variants were identified by PCR-RFLP methods, as described by van der Logt *et al*^[20].

Table 1 Clinical features of pancreatic cancer patients

Characteristic	Pancreatic cancer patients n = 61
Mean age ± SD (range) (yr)	63 ± 10 (44-81)
Male/Female	31 (50.8%)/30 (49.2%)
Primary tumor location	
Head	38 (62.3%)
Body	15 (24.6%)
Tail	8 (13.1%)
Smoking	
Yes/No	18 (29.5%)/43 (70.5%)

Briefly, to detect variations at *UGT1A7* codons 129/131, we used the forward primers F1 and F2 and the reverse primer R1 (Table 2). F1 only detects the N129K/R131K polymorphism, whereas F2 detects both the N129K/R131K and N129R/R131K polymorphisms. To detect the W208R alteration, we used the forward primer F3 and the reverse primer R2 (Table 2). To determine whether the N129K/R131K or N129R/R131K and W208R occur cis or trans, we used the primers F4 and R1 (Table 2). PCR conditions were 5 min at 95°C, then 37 cycles of 45 s at 95°C, 45 s at 55°C (codons 129/131)/65°C (codon 208)/56°C (allele specific), and 45 s at 72°C, and finally an elongation step at 72°C for 5 min. Aliquots of 10 µL of the PCR product were digested with the restriction enzyme *VspI* (codons 129/131) or *RsaI* (codon 208/allele specific) for at least 1 h at 37°C.

Genotyping of *UGT1A9* (MIM: 606434)

The strategy used to identify polymorphisms in the *UGT1A9* was PCR amplification of the exon 1, followed by direct DNA sequencing. *UGT1A9* exon 1 sequences were amplified using a 5' primer that specifically anneals to sequence upstream of the *UGT1A9* (GenBank accession number S55985) first exon, which is unique to the *UGT1A9* gene. Specific primers (Table 2) were used to amplify a PCR product of 939 bp^[21]. The PCR conditions were 3 min at 94°C, then 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 60 s. The *UGT1A9* was sequenced on both strands for all subjects. The sequence of the PCR products was determined by automated sequencing and analysed by using Sequencing Analysis 3.4.1 (Applied Biosystems, Applied Biosystems, Foster City, CA).

Genotyping of *ARP* (MIM: 601916)

PCR amplification of the (AGG) repeats around codon 50 of the *ARP* gene was performed using two primers, G and I (Table 2)^[9], and analyzed by both sequencing and genotyping assay. For genotyping assay, forward primer was labelled with fluorescent 6-FAM dye (provided by MWG-Biotech AG). Enzymatic amplification of DNA was performed using AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Applied Biosystems) and amplification cycles consisted of 10 min at 94°C, followed by 35 cycles run at 94°C for 45 s, 62°C for 75 s and 72°C for 45 s, and a final extension step of 72°C for 10 min. The PCR products were analyzed for sequence by using Sequencing Analysis 3.4.1, and for genotyping by using Genescan (Applied Biosystems, Applied Biosystems).

Table 2 Primers and restriction enzymes used for genotyping analyses

Gene	Primer (5'→3'; F = forward; R = reverse)	Restriction enzyme
<i>UGT1A7</i> codon 129 and 131	F1 AAT TGC AGG AGT TTG a TT AA ¹	<i>VspI</i>
	F2 AAT TGC AGG AGT TTG a TT A ¹	<i>VspI</i>
	R1 TTC AGA GGC TAT TTC TAA GA	
<i>UGT1A7</i> codon 208	F3 ATG CTC GCT GGA CGG CAC CAT TG	<i>RsaI</i>
	R2 TGC CGT GAC AGG GGT TTG GAG A	
<i>UGT1A7</i> allele specific	F4 ATT GCA GGA GTT TGT TTA AGG ACA	<i>RsaI</i>
	R1 TTC AGA GGC TAT TTC TAA GA	
<i>UGT1A9</i>	F CAGGTTTGTGCTGGTATTTCTCCCA	
	R GCGGATATCCATAGGCACTGGCTTTCCTGATGACA	
<i>ARP</i>	G GGCCGGGACTTGGAGGCGGTG	
	I CAGCACGCTCAGAGCCAG	

¹Bold 'a' in the primer sequence denotes site-directed mutagenesis for introduction of a *VspI* restriction site in the wild-type allele.

Biosystems, Applied, Foster City, CA).

Genotyping of *SPINK1* (MIM: 167790)

The *SPINK1* polymorphisms in exon 3 were determined by automatic sequencing. PCR-RFLP (restriction endonuclease: *TaaI*, MBI Fermentas, Germany)^[22] or PCR-CTPP (PCR with confronting two pair primers)^[23] assays were used to confirm the N34S and P55S variants.

Genotyping of *CFTR* (MIM: 602421)

The 31 most frequent mutations were examined by an oligonucleotide ligation assay (OLA kit, Applied Biosystems, Applied, Foster City, CA), whereas other seven mutations, frequently observed in Italian cystic fibrosis patients were examined by sequencing as previously described^[24].

Statistical analysis

The Hardy-Weinberg equilibrium was tested by using the Arlequin software^[25]. Comparison of allele and genotype frequencies of *UGT1A7*, *UGT1A9*, *ARP*, *SPINK1* and *CFTR* polymorphism was evaluated using the Monte Carlo approach with CLUMP program^[26] for the presence of multiple allele variants.

Univariate analysis was performed for tumor location, age at diagnosis and smoking habits by sorting patients according to their genotype distribution using the SPSS statistical package (SPSS v13 Inc. Chicago, IL, US). Continuous variable was expressed as means and standard deviations. Comparison of variables was performed with ANOVA method. Categorical variables were reported as percentage and comparison of them was performed by χ^2 -test or Fisher exact test when appropriate.

Sample size calculation to detect a significant difference between two allelic frequencies was carried out based on data reported by Ockenga *et al.*^[7]. The software package available as shareware on the World Wide Web at <http://statpages.org/proppowr.html> was used to determine the sample size. We hypothesized an allelic frequency of 20% and 35% (difference proportion rate: 15%) in controls and pancreatic cancer patients. Setting the power of the test at 80% with a significance level of 5% and assuming a relative sample size proportion of 0.6 between pancreatic

cancer patients and controls, we calculated a sample size of 198 alleles for controls and 119 alleles for patients. Consequently, we decided to recruit at least 100 controls and 60 pancreatic cancers into our study.

RESULTS

A summary of the demographic characteristics of the study population is shown in Table 1. In five subjects the diagnosis was made on clinical ground; the remaining 56 cases were confirmed histologically as ductal adenocarcinoma. In 62.3% of patients the tumor was located in the head of the pancreas, in 24.6% in the body, and in 13.1% in the tail at the time of diagnosis.

For each of the five genes, the distribution of the allele frequencies among the control population was found to fit with the assumption of the Hardy-Weinberg equilibrium ($P > 0.05$).

Three mutations (N129K, R131K, and W208R) combined into four different alleles (*1: wild type; *2: carrying N129K and R131K; *3: carrying N129K, R131K and W208R; and *4: carrying only W208R) were detected in the *UGT1A7* gene. *UGT1A7* genotyping did not show a significantly different distribution of previous alleles among patients compared to healthy controls except for the genotype *2*3 more commonly observed in healthy controls [21.0% *vs* 6.6%; CLUMP (T3): $\chi^2 = 6.05$, $P = 0.01$]. Nevertheless, after 1000 simulations with CLUMP this difference became insignificant ($P_c = 0.07$) (Table 3). Most notably, the frequency of the *UGT1A7**3 risk allele was comparable between patients (42.6%) and controls (42.0%) ($P = NS$).

Two variations (Y242X and D256N) combined into three different alleles (*1: wild type; *4: Y242X; *5: D256N) were detected in the *UGT1A9* gene. The distribution of the *UGT1A9**1, *UGT1A9**4 and *UGT1A9**5 alleles did not differ in PC patients and controls (Table 3). The variations were observed only in heterozygous state in both patients and controls.

In *ARP* gene a high frequency of genetic changes in the multiple arginine-coding-area such as M50R substitution (14.9% *vs* 11.1%), insertion of (AGG)_n (1.4% *vs* 1.1%), deletion of (AGG)_n (2.7% *vs* 4.4%) was observed in both

Table 3 Allele and genotype frequencies of *UGT1A7* and *UGT1A9* gene polymorphisms in pancreatic cancers and controls *n* (%)

Polymorphisms	Cases (<i>n</i> = 61)	Controls (<i>n</i> = 105)
<i>UGT1A7</i> (exon 1)		
Allele frequencies		
*1	54 (44.3)	74 (35.0)
*2	15 (12.3)	46 (22.0)
*3	52 (42.6)	89 (42.0)
*4	1 (0.8)	1 (1.0)
CLUMP (T1): $\chi^2 = 5.66$ (df = 3), <i>P</i> = 0.10		
CLUMP (T3-column used 2): $\chi^2 = 4.75$ (df = 1), <i>P</i> _c = 0.07 (uncorrected <i>P</i> = 0.03)		
Genotype frequencies		
*1*1	10 (16.4)	14 (13.3)
*1*2	7 (11.5)	12 (11.4)
*2*2	2 (3.3)	6 (5.7)
*1*3	26 (42.6)	34 (32.4)
*2*3	4 (6.6)	22 (21.0)
*3*3	11 (18.0)	16 (15.2)
*1*4	1 (1.6)	0 (0.0)
*3*4	0 (0.0)	1 (1.0)
CLUMP (T1): $\chi^2 = 9.43$ (df = 9), <i>P</i> = 0.21		
CLUMP (T3-column used 5): $\chi^2 = 6.05$ (df = 1), <i>P</i> _c = 0.07 (uncorrected <i>P</i> = 0.01)		
<i>UGT1A9</i> (exon 1)		
Allele frequencies		
*1	121 (99.2)	206 (98.0)
*5	1 (0.8)	2 (1.0)
*4	0 (0.0)	2 (1.0)
CLUMP (T1): $\chi^2 = 1.19$ (df = 2), <i>P</i> = 0.79		
Genotype frequencies		
*1*1	60 (98.4)	101 (96.2)
*1*5	1 (1.6)	2 (1.9)
*1*4	0 (0.0)	2 (1.9)
CLUMP (T1): $\chi^2 = 1.19$ (df = 5), <i>P</i> = 0.79		

PC and HC, respectively. Thus, we genotyped this variable site as a simple tandem repeat (STR) polymorphism. Seven different alleles (*1: 143 bp; *2: 146 bp; *3: 149 bp; *4: 152 bp; *5: 155 bp; *6: 158 bp; *7: 176 bp) were detected with wild type sequence corresponding to allele *3 and the other allele to either deletion or insertion of trinucleotide repeats. We did not observe significant difference in both allele and genotype frequencies of *ARP* between PC patients and HC (Table 4).

Direct sequencing of the coding exonic and flanking intronic sequence of the exon 3 of *SPINK1* detected mutations in 5 (4.1%) out of 61 patients. Four of them carried a heterozygous N34S mutation, and one patient had a heterozygous P55S mutation (Table 4). In 4 (2.0%) out of 105 controls two heterozygous mutations in both N34S and P55S (*P* = NS) were detected.

For the *CFTR*, only the $\Delta F508$ mutation was found in a heterozygous state in 3 patients with PC (prevalence rate 2.5%) and in 4 controls (3.8%) (*P* = NS) (Table 4).

The statistical analysis was also performed after sorting pancreatic cancer patients out according to the histological type of the tumor, age at diagnosis and smoking habit. No statistically significant difference in genotype distribution of gene polymorphisms was observed (data not shown).

Table 4 Allele and genotype frequencies of *ARP*, *SPINK1* and *CFTR* gene polymorphisms in pancreatic cancers and controls *n* (%)

Polymorphisms	Cases (<i>n</i> = 61)	Controls (<i>n</i> = 105)
<i>ARP</i>		
Allele frequencies		
*1	4 (3.3)	4 (2.0)
*2	4 (3.3)	7 (3.0)
*3	111 (91.0)	195 (93.0)
*4	1 (0.8)	0 (0.0)
*5	1 (0.8)	2 (1.0)
*6	1 (0.8)	0 (0.0)
*7	0 (0.0)	2 (1.0)
CLUMP (T1): $\chi^2 = 5.25$ (df = 6), <i>P</i> = 0.52		
Genotype frequencies		
*1*3	4 (6.6)	4 (3.8)
*2*3	4 (6.6)	7 (6.7)
*3*3	50 (82.0)	90 (85.7)
*3*4	1 (1.6)	0 (0.0)
*3*5	1 (1.6)	2 (1.9)
*3*6	1 (1.6)	0 (0.0)
*3*7	0 (0.0)	2 (1.9)
CLUMP (T1): $\chi^2 = 5.29$ (df = 27), <i>P</i> = 0.54		
<i>SPINK1</i> (exon 3)		
Allele frequencies		
wt	117 (95.9)	206 (98.0)
P55S	4 (3.3)	2 (1.0)
N34S	1 (0.8)	2 (1.0)
CLUMP (T1): $\chi^2 = 2.36$ (df = 2), <i>P</i> = 0.29		
Genotype frequencies		
wt/wt	56 (91.8)	101 (96.2)
wt/P55S	4 (6.6)	2 (1.9)
wt/N34S	1 (1.6)	2 (1.9)
CLUMP (T1): $\chi^2 = 2.40$ (df = 5), <i>P</i> = 0.30		
<i>CFTR</i>		
Allele frequencies		
wt	119 (97.5)	206 (98.0)
$\Delta F508$	3 (2.5)	4 (2.0)
CLUMP (T1): $\chi^2 = 0.11$ (df = 1), <i>P</i> = 1.0		
Genotype frequencies		
wt/wt	58 (95.1)	101 (96.2)
wt/ $\Delta F508$	3 (4.9)	4 (3.8)
CLUMP (T1): $\chi^2 = 0.11$ (df = 2), <i>P</i> = 1.0		

Moreover, no evidence of interaction between investigated genes was found.

DISCUSSION

The link between genetic polymorphisms of *UGT1A*, *UGT1A9*, *ARP*, *SPINK1* and *CFTR* genes and PC represent an attractive model combining genetic predisposition with environmental exposure. In the present study, we performed a simultaneous analysis of all these five genes in patients with sporadic pancreatic carcinoma. Our working hypothesis was that the predisposition to pancreatic cancer could be due to a gene-gene interaction.

Oxidative cellular injury is a prominent mechanism that could induce pancreatic inflammation and lead to genotoxicity and cancer. A major family of proteins that

play a role in cellular detoxification and defense are the UDP-glucuronosyltransferase, which are expressed in different tissues with xenobiotic contact and have been implicated in chemical carcinogenesis^[7,27]. Moreover, recent findings indicate that the presence of UGT polymorphisms may modify the risk of cancer development and reinforce the critical role of this pathway in the regulation of the biological activity of endogenous molecules and in determining the response to toxic chemicals^[7]. In the present study we found that the frequency of genetic polymorphisms in *UGT1A7* in PC patients was not different from those of healthy controls. In particular, the frequency of the *UGT1A7**3 allele encoding less active enzymes was 42.6% in pancreatic cancer compared to 42% in healthy controls, a figure comparable to that reported by Verlaan *et al.*^[8] in patients with pancreatic disease (35%-48%) and healthy controls (41%), thus suggesting that individuals bearing this allele are not at a higher risk of developing PC. Two polymorphisms of the *UGT1A9* gene (*UGT1A9**4 and *UGT1A9**5), described only in Japanese population^[28-30], were found at a similar frequency in PC and healthy controls (0%-1%).

ARP gene has been described as a possible oncogene responsible for PC. Although the functional role of the encoded protein is still unknown, the gene was found evolutionarily conserved^[9]. To examine the involvement of the *ARP* gene in exocrine PC, we screened genetic variations around multiple AGG repeats and STR polymorphism present in exon 1. We did not find a difference in mutation frequency between PC patients and controls, well in keeping with Tanaka *et al.*^[31], who found that the frequency for wild-type, M50R substitution, (AGG)₂ insertion, and AGG deletion amounted to 82%, 14%, 3% and 1%, respectively^[31]. No significant difference in allele and genotype frequencies between PC and controls for STR polymorphism were observed.

Recently, *SPINK1* polymorphisms and *CFTR* mutations have been associated with long standing chronic pancreatitis^[32,33]. *SPINK1* is a potent protease inhibitor that is thought to specifically inactivate intrapancreatic trypsin^[34]. Evidence suggests that *SPINK1* may act as a protective mechanism by preventing the activation of the pancreatic digestive enzyme cascade. Mutations in *CFTR* may disturb the subtle balance between proteases and their inhibitors by intrapancreatic acidification or by a defective apical trafficking of zymogen granules that might facilitate the intrapancreatic activation of digestive enzymes and result in pancreatitis^[35,36]. In the current study, we observed that the prevalence of the *SPINK1* N34S and P55S polymorphisms was similar between PC patients and healthy controls as previously reported in Caucasian sporadic pancreatic cancer groups^[37]. Regarding the frequency of the most common *CFTR* mutations, we found a similar prevalence of the $\Delta F508$ mutation in PC patients and HC, consistent with similar results reported by Malats *et al.*^[19].

To the best of our knowledge, this is the first study to evaluate the prevalence of all five gene polymorphisms potentially involved in patients with exocrine pancreatic cancer. The findings of this study suggest that these polymorphisms are possibly not implicated in pancreatic cancer risk, although this study was powered to detect

a difference proportion rate of at least 15% in allelic frequencies between controls and pancreatic cancer patients. Moreover, no gene-gene interaction or correlation with tumor location, age at diagnosis and smoking habit could be demonstrated. Further studies are needed to explore other polymorphisms involved in the metabolism of carcinogens and/or endogenous factors.

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Evaluation of esophageal function in patients with esophageal motor abnormalities using multichannel intraluminal impedance esophageal manometry

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Abstract

AIM: To evaluate the functional aspect of esophageal motility in healthy subjects and in patients who were referred for esophageal function testing using multichannel intraluminal impedance-esophageal manometry (MII-EM), and to assess the clinical utility of MII-EM.

METHODS: From September 2003 to January 2004, we performed the MII-EM on healthy volunteers and all the patients who were referred for esophageal function testing. Each patient received 10 liquid and 10 viscous swallows. We analyzed the results, the impedance and the manometric findings. Some of the subjects had additional ambulatory 24-h pH study performed to diagnose gastroesophageal reflux disease (GERD).

RESULTS: Among 89 studied subjects, the MII-EM findings showed normal esophageal motility in 50 (56.17%), ineffective esophageal motility (IEM) in 17 (19.10%), nutcracker esophagus in 7 (7.86%), achalasia in 4 (4.49%), and scleroderma esophagus in 11 (12.35%) cases. The completeness and the speed of bolus transit were in the order of nutcracker esophagus, normal manometry and IEM. Some of the swallows showing normal manometry and IEM had incomplete transit. In the achalasia and scleroderma esophagus, almost all the swallows had incomplete transit. The body amplitudes were higher for the swallows with complete transit than for the swallows with incomplete transit. There was not a significant difference in the manometric and impedance findings between the subjects with and without GERD.

CONCLUSION: MII-EM is a useful tool in assessing the

esophageal function in the patients having esophageal motility abnormality. The primary factors influencing the bolus transit are the amplitude of the esophageal body and normal peristalsis.

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Key words: Impedance manometry; Esophageal function; Motility; Bolus transit

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INTRODUCTION

Esophageal manometry has been considered the "gold standard" test for the evaluation of esophageal motility. Esophageal manometry allows physicians to assess peristalsis by using informations about the shape, amplitude and duration of the esophageal contraction, but it does not offer direct information about bolus transit, while barium study and single photon emission computed tomography visualize the bolus transit and offer anatomical information, but these techniques have the disadvantage of radiation exposure^[1].

The principles of impedance testing are based on measuring the differences in resistance to an alternating current that passes through the intraluminal contents. Impedance testing can detect and quantify bolus movement^[2-4]. The ability of impedance testing to detect bolus transit was validated with video-fluoroscopy in normal subjects^[5,6]. Combined multichannel intraluminal impedance-esophageal manometry (MII-EM) evaluates the functional aspects of esophageal contractions by simultaneously measuring bolus transit and esophageal contraction. Generally, the findings of esophageal manometry correlate with the symptoms or clinical progress in achalasia and diffuse esophageal spasm, but their findings do not always correlate in nutcracker esophagus and ineffective esophageal motility (IEM). The assessment of bolus transit and

esophageal function may help the physician to understand the clinical progress of a patient with esophageal disease. The aim of this study was to evaluate the functional aspects of esophageal contraction in patients having diverse esophageal motility abnormalities by using MII-EM, and to investigate its clinical utility.

MATERIALS AND METHODS

Impedance manometry

From September 2003 to January 2004, all the subjects who were referred for esophageal function testing and the healthy volunteers had esophageal function testing performed using MII-EM technology at Kangnam St. Mary's hospital, the Catholic university. The volunteers did not have any esophageal symptoms, as was investigated by an esophageal symptoms questionnaire, and they also did not have any gastrointestinal motility disorders that would influence esophageal motility, as was ascertained by physical examination and history taking. The MII-EM catheter (Sandhill EFT catheter; Sandhill Scientific Inc. Highland Ranch, CO.) was inserted transnasally into the esophagus. The 4.5-mm diameter catheter design had two circumferential solid-state pressure sensors at 5 cm and 10 cm from the tip, and it had two unidirectional pressure sensors at 15 cm and 20 cm from the tip. The impedance measuring segments consisted of pairs of metal rings placed 2 cm apart and they were centered at 10, 15, 20 and 25 cm from the tip. Intravesophageal pressure sensors and impedance measuring segments were thus located at 5, 10, 15 and 20 cm above the lower esophageal sphincter (LES), respectively (Figure 1). The LES was identified using the stationary pull-through method. The esophageal function test was performed after the subjects had rested for 10 min. With the patients in a sitting position, they were given 10 swallows of 5 cm³ normal saline and 10 swallows of 5 cm³ viscous material at 20-30 s apart. Normal saline was used instead of water since it has a standardized ionic concentration and provides for better impedance changes. The viscous material was manufactured as a food substance, and its known, standardized impedance value was provided by Sandhill Scientific Co.

Analysis

Manometry: The swallows were manometrically classified as (1) normal if the contraction amplitudes at 5 and 10 cm above the LES were each greater than or equal to 30 mmHg, and the distal onset velocity was less than 8 cm/s, (2) ineffective if either of the contraction amplitudes at 5 and 10 cm above the LES was less than 30 mmHg, (3) simultaneous if the contraction amplitudes at 5 and 10 cm above LES were each greater than or equal to 30 mmHg at the site, and the distal onset velocity was greater than 8 cm/s.

The manometric parameters used to characterize the swallows included (1) the contraction amplitude at 5 and 10 cm above the LES, (2) the distal esophageal amplitude as the average of the contraction amplitudes at 5 and 10 cm above the LES, and (3) the onset velocity of the esophageal contractions in the distal part of the esophagus. The mid-respiratory resting pressure and the LES residual pressure during swallows were used to assess the LES

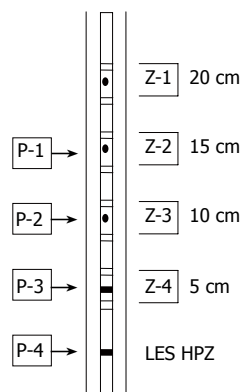


Figure 1 The 8-channel MII-EM catheter. Pressure sensors are located in LES high pressure zone (HPZ) (P-4), 5 cm (P-3), 10 cm (P-2) and 15 cm (P-1) above LES. Impedance measuring segments are centered on 5 cm (Z-4), 10 cm (Z-3), 15 cm (Z-2) and 20 cm (Z-1) above LES.

function.

Impedance: In the impedance curve, the bolus entry at each level was determined as the 50% point between the 3-s pre-swallow impedance baseline and the impedance nadir during the presence of the bolus, and the bolus exit was determined as the return to this 50% bolus point on the impedance recovery curve.

The swallows were classified by MII as showing (1) complete bolus transit, if the bolus entry occurred at the most proximal site (20 cm above LES) and the bolus exit points were recorded at all three distal impedance measuring sites (15, 10 and 5 cm above LES), (2) incomplete bolus transit, if the bolus exit points were not identified at any one of the three distal impedance measuring sites. We calculated the complete bolus transit rate for the liquid and viscous swallows.

Impedance parameters included (1) the total bolus transit time (TBTT) as the time that elapsed between bolus entry at 20 cm above the LES and bolus exit at 5 cm above the LES, (2) the complete bolus transit rate, and (3) the baseline impedance during the resting state. To assess the baseline impedance value, a pair of cursors (the time difference between the cursors was about 4 s) were placed on one channel about 2-3 s before the onset of the impedance changes related to the arrival of a bolus front. The maximal and minimal values between the cursors were subsequently determined by computer analysis. The baseline impedance was defined as the mean of these values.

Evaluation of esophageal motility and function: The diagnoses of manometric motility abnormalities were established from 10 liquid swallows with using the criteria published by Spechler and Castell^[7]. Achalasia was defined by the absence of esophageal body peristalsis and, if present, a poorly relaxing LES. Scleroderma esophagus was defined based on an appropriate clinical diagnosis and confirmed by the presence of low amplitude contractions in the distal esophagus with or without a low LES pressure. Diffuse esophageal spasm was defined as 20% or more simultaneous contractions. IEM was defined as 30% or more swallows with a contraction amplitude less than 30 mmHg in either of the two distal sites located at 5 and 10 cm above the LES. Nutcracker esophagus was defined as normal peristalsis of the esophageal body with the average distal esophageal amplitude exceeding 180 mmHg. Poorly relaxing LES was defined as the average LES residual pressure exceeding 8 mmHg and this was associated with nor-

mal esophageal body contractions. Hypertensive LES was defined as the LES resting pressure exceeding 45 mmHg with normal esophageal body contractions. Hypotensive LES was defined as the LES resting pressure below 10 mmHg with normal esophageal body contractions. Normal esophageal manometry was defined as not more than 20% ineffective swallows and not more than 10% simultaneous swallows with a distal esophageal amplitude < 180 mmHg and with normal LES resting and residual pressures. For the patients having both esophageal body and LES abnormalities, the final diagnosis was based on the esophageal body findings.

The overall diagnosis of esophageal transit abnormalities was defined as abnormal liquid transit if more than 20% of the liquid swallows had incomplete bolus transit and there was abnormal viscous transit if more than 30% of the viscous swallows had incomplete bolus transit.

Statistical analysis

Descriptive statistics were used to describe the manometric and impedance findings. Analysis of variance (ANOVA) was used to evaluate the differences of impedance parameters by the manometric diagnosis. Unpaired *t*-test was used to compare the differences of the distal esophageal amplitudes between swallows with and without complete transit. Unpaired *t*-test was also used in the comparison of the manometric and impedance parameters between the patients with and without gastroesophageal reflux. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Subjects

Using combined MII-EM, 89 subjects including 26 healthy volunteers (27 males and 62 females, mean age: 41.4 years), underwent esophageal function testing. While all the subjects received liquid swallows, 10 subjects did not receive the viscous swallows. The primary symptoms for which the subjects received esophageal function testing were: ENT symptoms, such as throat discomfort or a globus sense in 26 subjects, chronic cough in 5 subjects, heartburn in 16 subjects (including 6 scleroderma patients), dysphagia in 4 subjects, other symptoms in 4 subjects and there were asymptomatic 8 scleroderma patients in order to evaluate the esophageal involvement of scleroderma. In 37 subjects, esophageal function testing was performed prior to ambulatory 24-h pH monitoring, which was done to diagnose gastroesophageal reflux disease (GERD).

Based on the aforementioned manometric criteria of liquid swallows, 50 (56.17%) patients had normal esophageal manometry, 17 (19.10%) patients had IEM, 7 (7.86%) patients had nutcracker esophagus, 11 (12.35%) patients had scleroderma esophagus and 4 (4.49%) patients had achalasia. Two patients with nutcracker esophagus had hypertensive LES simultaneously. There was no patient who had an isolated LES abnormality (hypertensive LES, hypotensive LES and poorly relaxing LES). The additional viscous swallows did not change the diagnosis of achalasia, scleroderma esophagus and nutcracker esophagus. However, the manometric diagnoses of 8

Table 1 Impedance parameters of the liquid and viscous swallows by the manometric diagnosis (mean \pm SD)

Impedance parameter	Bolus transit rate (%)		TBTT (s)	
	Liquid	Viscous	Liquid	Viscous
Normal	88 \pm 18	72 \pm 28 ^d	6.5 \pm 1.3	7.6 \pm 1.5 ^d
IEM	59 \pm 16 ^b	61 \pm 27 ^{b,d}	7.4 \pm 1.6 ^b	8.3 \pm 1.4 ^{b,d}
Nutcracker esophagus	98 \pm 8 ^b	98 \pm 5 ^{b,d}	6.0 \pm 1.2 ^b	6.9 \pm 1.4 ^{b,d}
Scleroderma	9 \pm 14 ^b	3 \pm 5 ^b	-	-
Achalasia	5 \pm 10 ^b	0 ^b	-	-

^b*P* < 0.01, *vs* normal by manometric diagnosis upon liquid swallows and viscous swallows (ANOVA); ^d*P* < 0.01, *vs* liquid swallows (unpaired *t*-test).

among the 50 subjects who had normal manometry upon the liquid swallows were changed to IEM, and 4 among the 17 patients who had IEM upon the liquid swallows had their diagnosis changed to normal manometry upon the viscous swallows when we applied the same manometric criteria to the patients receiving viscous swallows. The final manometric diagnosis for each patient was based on the liquid swallows so as to remain consistent with the current tradition.

Evaluation of overall bolus transit using liquid and viscous swallows

The bolus transit rate was high and the TBTT was short in the order of nutcracker esophagus, normal manometry and IEM, upon both liquid and viscous swallows (^b*P* < 0.01). The liquid bolus moved faster than the viscous bolus (^d*P* < 0.01, Table 1).

All patients with nutcracker esophagus had normal transit for the liquid and viscous swallows. For the subjects with normal manometry, 20% had abnormal liquid transit and 34% had abnormal viscous transit, and 46% of the patients with IEM had abnormal viscous transit. None of the patients with achalasia and scleroderma esophagus had normal transit for the liquid and viscous swallows.

In the analysis of individual swallows, the liquid bolus transit rates were 90% for the manometric normal swallows, 24% for the manometric ineffective swallows and 5% for the manometric simultaneous swallows. The viscous bolus transit rates were 83% for the manometric normal swallows, 9% for the manometric ineffective swallows, and 0% for the manometric simultaneous swallows (Table 2).

The distal esophageal amplitudes of the swallows with complete transit were significantly higher than those of the swallows with incomplete transit for both the liquid and viscous swallows, except for the manometric simultaneous swallows (*P* < 0.01, Figure 2).

Baseline impedance

The baseline impedance values for the patients with achalasia and scleroderma esophagus were lower than those of the subjects with normal manometry, IEM and nutcracker esophagus at all impedance measuring sites (*P* < 0.05, Table 3). There was no difference in the baseline impedance among all of the impedance-measuring sites for the patients with achalasia, but for the scleroderma patients, the impedance of the most proximal site was

Table 2 Manometric and impedance evaluation of the liquid and viscous swallows

		Manometric evaluation					
		Normal		Ineffective		Simultaneous	Total
		<i>n</i>	<i>r</i> %	<i>n</i>	<i>r</i> %	<i>n</i>	<i>r</i> %
MII evaluation							
Liquid							
Complete transit	<i>n</i>	578	91.6	51	8.1	2	0.3
	C%	89.6		23.7		4.9	
Incomplete transit	<i>n</i>	67	24.8	164	60.7	39	14.4
	C%	10.4		76.3		95.1	
Total	<i>n</i>	645	71.6	215	23.9	41	4.6
Viscous							
Complete transit	<i>n</i>	423	96.1	17	3.9	0	0
	C%	82.5		9.1		0	
Incomplete transit	<i>n</i>	90	29.6	170	55.9	44	14.5
	C%	17.5		91.9		100	
Total	<i>n</i>	513	69.0	187	25.1	44	5.9

r%; Row percent (i.e. percent with given manometric evaluation); *c*%: Column percent (i.e. percent with given impedance evaluation).

Table 3 Baseline impedance by the manometric diagnosis (mean ± SD)

Manometric diagnosis	Impedance measuring site (distance from the LES)			
	20 cm (Ω)	15 cm (Ω)	10 cm (Ω)	5 cm (Ω)
Liquid				
Normal	1530 ± 549 ^a	1353 ± 464 ^a	1808 ± 608 ^a	2440 ± 778 ^a
IEM	1430 ± 572 ^a	994 ± 350 ^a	1318 ± 507 ^a	1875 ± 757 ^a
Nutcracker	1329 ± 539 ^a	1348 ± 550 ^a	1636 ± 712 ^a	2132 ± 1089 ^a
Scleroderma	1006 ± 503	448 ± 238	419 ± 194	402 ± 195
Achalasia	658 ± 478	456 ± 287	391 ± 140	660 ± 465
Viscous				
Normal	1415 ± 494 ^a	1317 ± 428 ^a	1589 ± 549 ^a	2518 ± 1452 ^a
IEM	1324 ± 476 ^a	1050 ± 289 ^a	1269 ± 465 ^a	1849 ± 742 ^a
Nutcracker	1447 ± 127 ^a	1302 ± 220 ^a	1642 ± 285 ^a	2206 ± 342 ^a
Scleroderma	787 ± 418	403 ± 160	378 ± 158	331 ± 145
Achalasia	388 ± 237	335 ± 150	323 ± 59	528 ± 343

^a*P* < 0.05, comparison between scleroderma esophagus or achalasia and the others; *P* values are not significant among normal manometry, IEM and nutcracker esophagus at all the impedance measuring sites in both liquid and viscous swallows (ANOVA).

markedly higher than those of the other sites (*P* < 0.01).

MI-EM in the patients with pathologic gastroesophageal reflux

Among the 37 subjects who had a 24-h pH study performed, 26 (70.27%) had normal results and the 11 (29.72%) subjects were diagnosed with GERD. The manometric diagnoses for the subject without pathologic gastroesophageal reflux were normal in 62%, IEM in 23% and nutcracker esophagus in 15%. The manometric diagnoses for the subjects with pathologic gastroesophageal reflux were normal in 73%, IEM in 18% and nutcracker esophagus in 9% cases. The distal esophageal amplitudes of the patients without pathologic reflux were higher than those of the subjects with pathologic gastroesophageal reflux (*P* < 0.05). The LES pressure, the pattern of esophageal contraction, bolus transit rate and the 'TBT' were not obviously different between them (Table 4).

Table 4 The manometry and impedance evaluation based on pathologic gastroesophageal reflux (mean ± SD)

	Swallow	Pathologic reflux (+)	Pathologic reflux (-)	<i>P</i>
Manometry				
LES pressure (mmHg)	All	21.9 ± 9.5	28.8 ± 9.5	NS
Distal esophageal amplitude (mmHg)	Liquid	113.6 ± 60.6	133.8 ± 78.5 ^a	< 0.05
	Viscous	113.2 ± 69.7	134.1 ± 79.1 ^a	< 0.05
Impedance				
TBT (s)	Liquid	6.7 ± 1.5	6.6 ± 1.5	NS
	Viscous	7.0 ± 1.0	7.1 ± 1.6	NS
Bolus transit rate (%)	Liquid	91 ± 14	86 ± 16	NS
	Viscous	73 ± 21	70 ± 30	NS

^a*P* < 0.05 vs pathologic positive reflux.

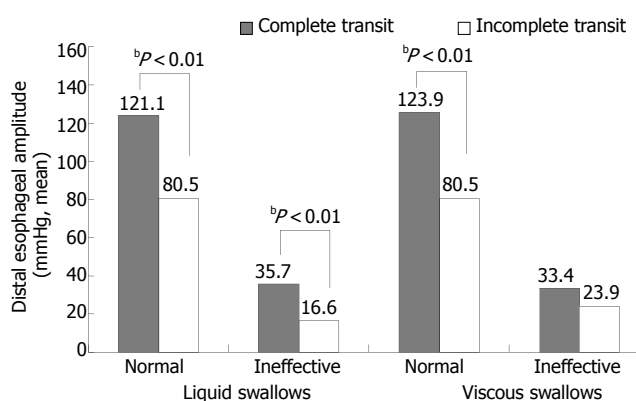


Figure 2 Comparison of the distal esophageal amplitudes between swallows with and without complete transit in the manometric normal and ineffective swallows (unpaired *t*-test). ^b*P* < 0.01, comparison between swallows with and without complete transit.

DISCUSSION

We evaluated the peristalsis and bolus transit in the subjects having abnormal and normal esophageal motility by using combined MII-EM. All the patients with achalasia and scleroderma esophagus, and some of the patients with IEM had abnormal bolus transit. Yet the patients with nutcracker esophagus had much better bolus transit than did the subjects having normal manometry.

The subjects having diverse esophageal motility abnormalities as well as the asymptomatic volunteers were included. Especially, the eleven patients with typical scleroderma esophagus were included. Most of the manometric ineffective swallows belonged to the scleroderma esophagus group and the IEM group. The bolus transit rate was markedly different between the IEM and scleroderma esophagus: 59% vs 9% on the liquid swallows and 61% vs 3% on the viscous swallows, respectively. Almost all the manometric simultaneous swallows belonged to the achalasia group. The bolus transit rate of the manometric simultaneous swallows was very low, less than 5%. We propose that the manometric ineffective or simultaneous swallows of patients with severe esophageal dysmotility and those of patients with the mild motility abnormality or normal motility should be classified and analyzed separately because their bolus transits were markedly different, as was

seen by the results.

The esophageal body pressure, the LES pressure and the peristaltic movement can be considered as factors that have an influence on the bolus transit. This study showed that the major determinants of complete bolus transit were the esophageal body pressure and normal peristalsis. The bolus transit rate and the TBTT were better in the order of nutcracker esophagus, normal manometry and IEM. The higher the distal esophageal amplitude was, the better was the bolus transit. The contraction amplitudes of the swallows with complete transit were higher than those of the swallows with incomplete transit. So, it can be concluded that the esophageal body pressure is important to determine the complete bolus transit. Because there were no subjects with a poorly relaxing LES or hypotensive LES and only two patients with hypertensive LES in this study, we could not evaluate the effect of LES pressure on bolus transit. As the bolus transit rate and the TBTT of the two subjects with hypertensive LES who were classified as nutcracker esophagus were not different from those of the other subjects with nutcracker esophagus and normal LES pressure, we can guess that the high esophageal body amplitude overcomes the interference with bolus transit by the hypertensive LES. Almost all the swallows of the patients with achalasia and scleroderma esophagus had functional aperistalsis and incomplete bolus transit, so normal peristalsis is very important for bolus transit. If the subjects with diffuse esophageal spasm were included in this study, we could then know the role of isolated peristaltic abnormality on bolus transit. Our conclusion somewhat coincides with Tutuian *et al.*^[8] that the major determinant of bolus transit is the esophageal body pressure and any isolated LES pressure abnormality has a minor role. They suggested the new functional classification of esophageal motility abnormality: defects of bolus transit and isolated pressure abnormalities. The defects of bolus transit include achalasia, scleroderma esophagus, IEM and diffuse esophageal spasm. The isolated pressure defects include nutcracker esophagus, hypertensive LES, hypotensive LES and poor relaxation of the LES.

We suggest that the nutcracker esophagus should be classified as functionally normal as it had a better bolus transit than the manometrically normal esophagus. Up to now, esophageal hypercontraction abnormalities, such as hypertensive LES and nutcracker esophagus, have been the most controversial of the dysmotility patterns because it is not clear that esophageal hypercontraction has any physiological importance. Achalasia clearly showed low baseline impedance and failure of bolus transit. Retrograde esophageal contraction, intermittent reflux of the luminal contents and pathologic movement of luminal air during swallows seen in a previous study^[9] were not clearly observed. Almost all the swallows of the patients with a scleroderma esophagus, in which the peristalsis was present faintly but very hypotensive, had incomplete transit like the achalasia patients. Because impedance testing has not yet been validated with radiographic studies in these patients, we should consider that impedance testing might overestimate incomplete transit. For example, the low pre-swallow impedance caused by the residual luminal content may influence the analysis of impedance of the following

swallow.

There was no difference of baseline impedances among the patients with normal manometry, IEM and nutcracker esophagus. Compared to those, the baseline impedances of swallows with achalasia and scleroderma esophagus were significantly lower. Impedance correlates negatively with the cross sectional area of the esophagus and the conductivity of the luminal contents^[10]. Low baseline impedance means the dilatation of lumen or the existence of luminal content. So, we could expect that the baseline impedance of the achalasia patients was significantly lower^[11-13]. We had expected that impedance of the distal esophagus in the patients with achalasia would be higher than the impedance at the more proximal sites, but it was not. In the scleroderma esophagus, the impedance of the proximal esophagus was higher than that of the distal esophageal body. It means that the bolus transit of the upper esophagus and around the UES is preserved compared to the other sites. The low impedance of the distal esophagus adjacent to LES seemed to be associated with the decreased resistance of the gastroesophageal junction, which consists of smooth muscle^[14]. The baseline impedance is considered to be the reflection of the dilatation of the esophageal body or the residual bolus produced by aperistalsis rather than it being a factor influencing bolus transit.

The bolus transit rate in the subjects with normal manometry was lower and had wider variation, as compared to previous studies. For example, one patient with normal manometry had a 20% transit rate for both liquid and viscous transit. Another patient with normal manometry had 100% liquid transit, but 0% viscous transit. The only 85% of the asymptomatic subjects with normal manometry had normal liquid transit and 78% had normal viscous transit. This showed that the precise evaluation of esophageal function was impossible via the indirect information from manometry only.

The viscous material may be a more sensitive material for the detection of minor motility abnormalities because generally, the viscous transit rate and transit time were worse than the liquid transit rate and transit time in the manometric normal and ineffective swallows. We should also consider the results of viscous swallows for the evaluation of esophageal dysmotility in that the manometric diagnoses of some patients with minor IEM or normal manometry were changed after applying the result of viscous swallows.

Some of the manometric ineffective and simultaneous swallows had complete transit, and there is the possibility of overestimation of the functional defect when using only the parameters of the manometry. The functional information from MII-EM will be useful for understanding the symptoms or pathophysiology of esophageal dysmotility which is minor or of unclear clinical significance such as nutcracker esophagus. However, the use of MII-EM is not always needed in daily clinical situation in that its result does not change the treatment option.

Impedance did not offer any additional information for the diagnosis of GERD. The differences of the distal esophageal amplitudes may be due to the fact that the group of patients without reflux included more number of

patients with nutcracker esophagus. We had expected that the bolus transit of patients with pathologic gastroesophageal reflux would be worse because they had the abnormal acid clearance, but their parameters of impedance were not significantly different from those of the patients who were without gastroesophageal reflux.

In conclusion, the combined MII-EM provides delicate and functional informations about the bolus transit of normal subjects and the patients with minor esophageal dysmotility as well as severe esophageal dysmotility. The major factors to determine the complete bolus transit are the esophageal body pressure and normal peristalsis. The validation of measuring impedance along with performing radiographic study will be needed for the patients with esophageal motility abnormality, and its clinical and prognostic value should be clarified by an outcome study^[15].

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Expression of 5-Lipoxygenase in human colorectal cancer

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Abstract

AIM: To evaluate the 5-lipoxygenases (Loxs) expression level in human colorectal cancer specimens in order to determine its clinicopathologic significance in human tumorigenesis.

METHODS: The relative quantity of 5-Lox mRNA in paired 91 colorectal tumor and adjacent normal mucosa samples was determined by real time quantitative PCR. Additionally, the expression of 5-Lox and cyclooxygenase (Cox)-2 proteins was also examined using immunohistochemical staining methods.

RESULTS: There was a marked increase in 5-Lox mRNA levels in the tumor compared with paired normal mucosa samples ($P < 0.0001$). Sixty six (72.5%) tumors showed high 5-Lox mRNA levels. The positivity rate of 5-Lox and Cox-2 protein expression was 68.7% and 79.1% respectively. There was a significant association between tumoral 5-Lox mRNA level and tumor size ($Rho = 0.392$, $P = 0.0002$), depth or vessel invasion.

CONCLUSION: These results suggest that 5-Lox is up-regulated in colorectal cancer and that inhibition of its expression might be valuable in the prevention and treatment of colorectal cancer.

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Key words: Arachidonic acid; 5-Lipoxygenase; Cyclooxygenase-2; Real time PCR; Immunohistochemistry; Colorectal cancer

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INTRODUCTION

Evidence from epidemiological and animal studies suggests that a high-fat consumption is associated with an increased incidence and growth of tumors at several specific organ sites, including breast, pancreas, colon, and prostate^[1]. In this respect, it has been demonstrated that the metabolism of arachidonic acid, a polyunsaturated fatty acid by either the Cyclooxygenase (Cox) pathway or the Lipoxygenase (Lox) pathway, generates a host of pro-inflammatory substances called eicosanoids including prostaglandins, thromboxanes, leukotrienes, *etc.*, that act as potent autocrine and paracrine regulators of cell biology^[2]. These substances are known to modulate diverse physiological and pathological responses, including growth and invasiveness of tumor cells as well as suppression of immune surveillance^[2].

To date, two Cox isozymes have been identified, the constitutive Cox-1, and the inducible Cox-2^[3-5]. For Lox, three major isoforms have been characterized in human tissue, according to the oxygenation sites in the substrate arachidonic acid. These enzymes are named, 5-, 12-, and 15-Lox^[4-7]. Over the last two decades, a large body of evidence from experimental and clinical studies has shown that Cox-1/-2 play an important role in the promotion and progression of many types of human neoplasms^[3,8-11]. And most importantly, specific inhibition of these enzymes is reported to exhibit a dramatic anti-neoplastic activity^[12-15]. Although most attention has focused on prostaglandins (PGs) and other Cox-derived metabolites, emerging evidence now suggests that Lox-catalyzed products, Leukotrienes (LTs) and Hydroxyeicosatetraenoic acids (HETEs) also exert profound biological effects on the development and progression of human cancers^[7]. An increase in expression of Lox and their metabolites has been detected in a variety of human cancer cell lines and tissues including those of the prostate^[16], bladder^[17], breast^[18], lung^[18], colon^[5,19], pancreas^[1,20], oral^[21] and esophagus^[22], and this over-expression was reported to be significantly associated with tumor cell proliferation, resistance to apoptosis, and angiogenesis^[23-25]. Moreover, the direct inhibition of 5-Lox or 12-Lox, was found to markedly suppress the tumor cell growth^[2,17,18,26]. Nonetheless, some investigators also have demonstrated a

down-regulation of 5-Lox-1/-2 in human colorectal^[27,28] and prostate tumors^[29] compared with adjacent normal tissues. The clinicopathologic significance of Lox expression is less clearly defined in human colorectal cancer. This observation prompted us to evaluate the expression level of 5-Lox and, determine its correlation with clinical and pathological features of colorectal cancer.

MATERIALS AND METHODS

Patients and tissue samples

Ninety one patients who underwent surgical resection for colorectal cancer in our institution between January and December 2004 were included in this study. These consisted of 53 men and 38 women with a mean age of 64 ± 11 years; 49 colon cancers and 42 rectal cancers. The clinicopathologic parameters were determined according to the International Union Against Cancer Tumor-Node-Metastasis (TNM) classification of malignant tumors^[30] and, the tumor size represents the greatest dimension measured after formalin fixation. Immediately after surgery, a small piece of colorectal tumor samples and matched adjacent normal mucosa (taken from the borders of the surgical specimen), were separately placed directly in RNA stabilization reagent (RNAlater, Qiagen) and stored frozen at -80°C until further analysis. Additionally, 67 formalin-fixed, paraffin-embedded tumor tissue blocks obtained from the same patients were also available for immunohistochemical studies.

This study was approved by the Institutional Review Board of the Tokyo Medical and Dental University, and written informed consent was obtained from all patients.

RNA extraction and cDNA synthesis

Total RNA for each sample was extracted using the RNeasy mini kit (Qiagen) according to the manufacturers' instructions. RNA was quantified by measurement of A_{260} and A_{280} using a UV spectrophotometer (Beckman, Life Science); and, its quality was determined by electrophoresis through agarose gels and visualization of the 18S and 28S RNA bands under UV light. For cDNA synthesis, 10 μg of total RNA was reverse-transcribed into cDNA samples using Superscript II RNase H-Reverse transcriptase (Invitrogen) according to the manufacturer's protocol.

Real time PCR

Real time quantitative Polymerase Chain Reaction (RTQ-PCR) analysis was done by using the 7300 Real Time PCR System (Applied Biosystems). Primers and probe sequences were designed using the Primer Express software (Applied Biosystems) as follows: 5-Lox primers: GGGCATGGAGAGCAAAGAAG and ACCTCGGCCGTGAACGT, probe: TACTTCTACCGGGACGACGGGCTCCT; ACTB-1 primers: TGAGCGCGGCTACAGCTT and TCCTTAATGTCACGCACGATTT; probe: ACCACCACGGCCGAGCGG.

The PCR reaction was carried out with the TaqMan Universal PCR Master Mix (Applied Biosystems) using 1 μL of cDNA in a 24 μL final reaction volume. The thermal cycling conditions were as follows: 50°C for 2 min,

95°C for 10 min and 45 cycles of 15 seconds denaturation at 95°C and 1 min annealing at 60°C . Each sample was run in duplicate for both target gene and endogenous gene.

The amount of 5-Lox (target) normalized to ACTB-1 (endogenous control) and relative to HCT15 (calibrator), was determined by the comparative C_T method^[31] using the Relative Quantification (ddCt) Study Software (7300 Sequence Detection System version 1.2.1, Applied Biosystems).

The human colon adenocarcinoma cell line HCT15 used as a calibrator in this study, was kindly provided by Cell Resource Center for Biomedical Research Institute (Tohoku University, Miyagi, Japan).

Immunohistochemical evaluation of 5-Lox expression

A Universal Immuno-enzyme Polymer method (Nichirei simple staining) was performed for immunohistochemical staining as we previously described^[32]. Anti-5-Lox polyclonal antibody (Cayman Chemical, Ann Arbor, MI; dilution: 1:250) and anti-Cox-2 monoclonal antibody (Cayman Chemical, Ann Arbor, MI; dilution: 1:250) were used as the primary antibody for 2 h incubation at room temperature.

The immunoreactivity of 5-Lox/Cox-2 protein in the tumor cells was determined according to the procedure previously described^[32]. Briefly, staining intensity was scored as 0 (negative), 1 (weak), 2 (medium), 3 (strong). Extent of staining was scored as 0 (0%), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (76%-100%) according to the percentage of positive staining area in relation to the whole carcinoma area. Then, the sum of intensity and extent score was regarded as the final staining score for 5-Lox or Cox-2; and, tumors having a final score of ≥ 3 were considered to be positive^[32].

Statistical analysis

Median value of normal samples was used as the cut-off point to distinguish between low and high levels of mRNA expression. Nonparametric Wilcoxon and Mann-Whitney U tests were used to evaluate differences in 5-Lox mRNA expression levels between groups. The correlations with clinicopathologic parameters were assessed with a Chi-square test for categorical variables and a Spearman rank test for continuous variables. The strength of association between 5-Lox and Cox-2 staining scores was determined by the Spearman rank test. The difference was considered significant at $P < 0.05$. All analyses were performed with the statistical software package Stat View (version 5.0).

RESULTS

5-Lox mRNA expression and its association with clinicopathologic parameters

The median value of 5-Lox mRNA expression (relative quantity, RQ) in normal and tumor samples was 36.094 (minimum: 5.966, maximum: 131.859) and 61.113 (minimum: 6.228, maximum: 224.773), respectively. Both Wilcoxon and Mann-Whitney U tests, showed a significant difference in mRNA levels between the two groups of samples ($P < 0.0001$; Figure 1). Using the median RQ of normal samples as the cut off point, we found that 66 of

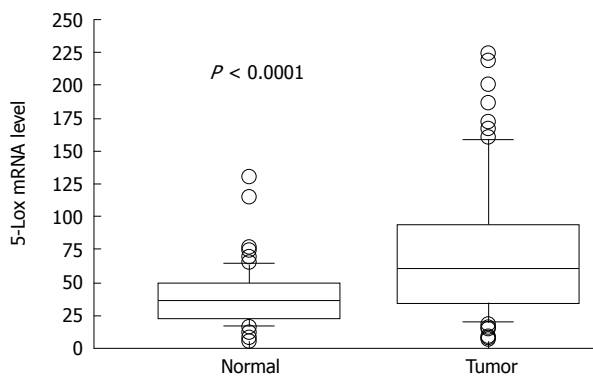


Figure 1 Differential 5-Lox mRNA relative quantity between matched normal mucosa and tumor tissue samples. Boxes: lower and upper quartiles (median inside); bars: at end of whiskers; individual plots: values outside the range of the whiskers.

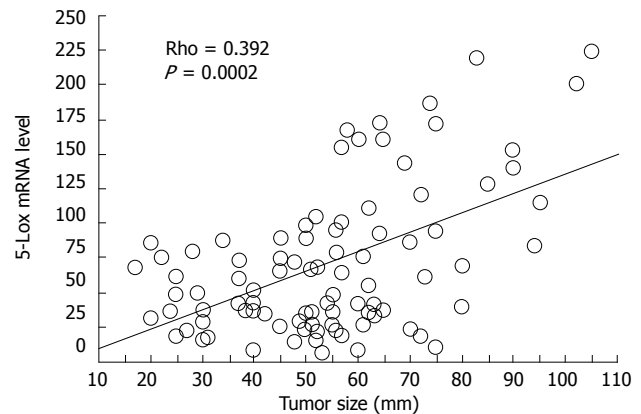


Figure 2 Linear regression plot showing a significant positive correlation between tumoral 5-Lox mRNA level and tumor size.

Table 1 Correlation of clinicopathologic parameters with 5-Lox mRNA expression

Clinicopathologic parameters	n	5-Lox expression		P
		Low	High	
All cases:	91	25 (27.47%)	66 (72.53%)	
Age:	91	Rho = 0.018		NS ¹
Gender:				
Male	53	15	38	NS
Female	38	10	28	
Tumor site:				
Colon	49	13	36	NS
Rectum	42	12	30	
Tumor size:	91	Rho = 0.392		0.0002 ¹
Histology:				
Well	49	11	38	NS
Moderate	34	13	21	
Poor	8	1	7	
Depth of invasion:				
pT1	11	7	4	0.021
pT2	18	6	12	
pT3	43	9	34	
pT4	19	3	16	
Lymph node metastasis:				
Yes	45	16	29	NS
No	46	9	37	
Lymphatic invasion:				
Yes	66	14	52	0.030
No	25	11	14	
Venous invasion:				
Yes	72	15	57	0.006
No	19	10	9	
TNM stage:				
I	12	2	10	NS
II	31	7	24	
III	38	15	23	
IV	10	1	9	

¹Spearman rank test P value; NS: Not Significant ($P > 0.05$).

91 tumor specimens (72.5%) showed high 5-Lox mRNA level. In Table 1, the associations between 5-Lox mRNA expression status and various clinicopathologic parameters are shown. 5-Lox expression was significantly correlated

with tumor size (Rho = 0.392, $P = 0.0002$; Figure 2), depth of invasion ($P = 0.0208$), lymphatic invasion ($P = 0.0297$), and venous invasion ($P = 0.0057$). There was no significant correlation with patient age and gender, tumor site, histological type, lymph node metastasis and TNM stage.

5-Lox and Cox-2 protein expression

Immunoreactivity of 5-Lox protein was found in the tumor epithelial cells within the cytoplasm, nucleus and nuclear membrane. In contrast, Cox-2 expression was mainly cytoplasmic. Occasionally, little staining was found in the stromal cells or tissue and the adjacent normal mucosa particularly for 5-Lox. Representatives of the staining patterns are shown in Figure 3.

The positivity rate (staining score of 3-7) for 5-Lox and Cox-2 expression was 68.7% and 79.1%, respectively. The Spearman rank test, showed a significant correlation between 5-Lox and Cox-2 staining scores (Rho = 0.277, $P = 0.0246$); and, 5-Lox mRNA levels versus protein staining scores (Rho = 0.380, $P = 0.0025$). Forty four samples stained concurrently positive for both proteins, and had a greater mean tumor size when compared with the group of other samples (58.273 ± 19.966 vs 46.391 ± 20.221 , $P = 0.0476$; Figure 4).

DISCUSSION

In the current study, we demonstrated that 5-Lox expression is significantly up-regulated in human colorectal tumor compared with the adjacent normal mucosa. Similar results have been reported for various tumors including those of the colon and rectum^[19], esophagus^[22], pancreas^[1], oral^[21], bladder^[17], and prostate^[16]. However, according to a PubMed search using the keywords 5-Lipoxygenase, and cancer, this is the first study to examine the expression levels and clinicopathologic significance of this gene in a large series of surgical specimens.

Our most important finding was the positive correlation found between 5-Lox expression level with tumor size, depth and vessels invasion. This observation is similar to our previous data indicating that gastric^[33] or colorectal^[32] tumors with higher Cox-2 level, grow

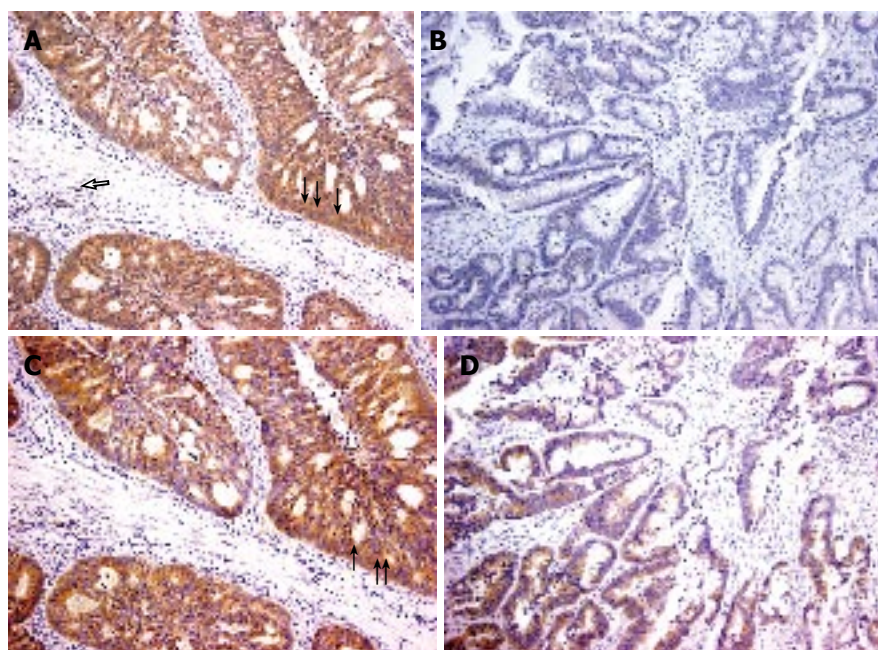


Figure 3 Representative cases of 5-Lox/Cox-2 immunostaining in colorectal cancer (x 200). **A:** Positive, diffuse, strong cytoplasmic and focal nuclear membrane 5-Lox staining in epithelial tumor cells (black arrow) and stromal cells (white arrow); **B:** Negative, weak 5-Lox staining. The same samples are shown with strong (**C**) and moderate (**D**) Cox-2 staining.

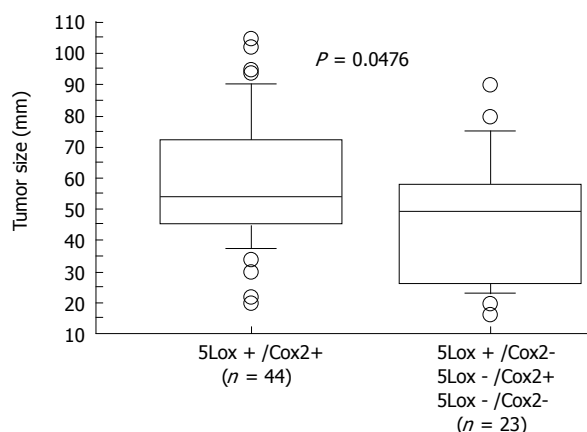


Figure 4 Differential tumor size between a group of concurrent 5-Lox/Cox-2 positive samples and a group of others (5Lox+/Cox2-; 5Lox-/Cox2+; 5Lox-/Cox2-).

more larger and deeper. It also provides further support to the concept that 5-Lox and Cox-2 display similarities in expression and function in human cancer such as proangiogenic and anti-apoptotic properties^[3] and a substrate preference. In our series, the fact that concurrent 5-Lox and Cox-2 positive tumors had the largest mean size, may be considered as the consequence of a combined effect of the two metabolic pathways that remove a proapoptotic substrate, arachidonic acid^[9], and generate more proangiogenic, anti-apoptotic products, eicosanoids; leading to an increase tumor cells growth. The absence of a statistically significant correlation between 5-Lox overexpression and more aggressive tumor behavior such as lymph node metastasis, confirmed the observation by Ohd *et al*^[19] that positive 5-Lox expression was not a strong predictor of survival in a study of 61 colorectal cancer patients^[19].

In contrast to Cox-2, little is known about the mechanisms by which lipoxygenases contribute to tumor growth and progression; but, emerging evidence from

several experimental studies suggest that these enzymes and their products, similarly to Cox-2 and PGs, may act on tumor cells through inhibition of apoptosis, increase of cell proliferation and stimulation of angiogenesis^[23-25]. Recently, Hoque *et al*^[25] using esophageal cancer cell lines, demonstrated that 5-Lox inhibitors caused a dose- and time-dependant reduction in cell viability and induced apoptosis, which was associated with the level of 5-Lox expression and LTB4 production in these cell lines^[25]. According to Romano *et al*^[24], the regulation of vascular endothelial growth factor (VEGF) release and mRNA levels by 5-Lox activity in malignant mesothelial cells was a crucial mechanism of 5-Lox actions on proliferation and apoptosis^[24]. Similarly, Ye *et al*^[34] in a mice inflammation-associated colon cancer formation model study, found that 5-Lox expression was accompanied by an up-regulation of matrix metalloproteinase (MMP)-2 activity and VEGF expression, both of which are key angiogenic factors for tumorigenesis^[34].

Regarding other Lox family enzymes, little is known about 12-Lox and, a great controversy exists in the literature as to the involvement of 15-Lox in human carcinogenesis. Ikawa *et al*^[5] reported that 15-Lox-1 was highly expressed in human colorectal tumor compared with adjacent normal mucosa^[5]. On the contrary, other investigators have extensively demonstrated not only a down-regulation, but also potential tumor-suppressor functions of 15-Lox-1/-2 in colorectal^[27,28] and prostate^[29] tumors.

Nevertheless, many questions still remain unanswered, including the exact contribution of individual enzymes as well as their interactions. Further studies are warranted. Finally, it is worthy to notice that several studies have consistently indicated that pharmacologic agents that specifically inhibit the Lox and/or Cox metabolic pathways, exhibit a dramatic anti-neoplastic activity in addition to their classic anti-inflammatory effect^[7,12-15,35]. And, despite the recent withdrawal of Vioxx (rofecoxib) from the

market and warning about other Cox-2 inhibitors over risk of cardiovascular side effects^[36], these pathways still remain an exciting and promising research area for cancer chemoprevention and treatment. Particularly the class of dual 5-Lox/Cox inhibitors such as licoferone (previously named ML3000) has emerged as an effective and well-tolerated therapy that could offer safety advantages over Cox inhibition alone^[4,37,38].

In conclusion, our data suggest that 5-Lox overexpression may influence the development of colorectal cancer. Therefore, inhibition of this metabolic pathway might provide an effective therapeutic option for colorectal cancer prevention and treatment.

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Inducible nitric oxide synthase polymorphism is associated with the increased risk of differentiated gastric cancer in a Japanese population

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in a Japanese population. This polymorphism may play an important role in increasing the risk of gastric cancer in Asian countries with the highest rates of gastric cancer.

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Abstract

AIM: To examine the association of inducible nitric oxide synthase (iNOS) C150T polymorphism with gastric cancer, as well as with gastric atrophy and *H pylori* seropositivity.

METHODS: A single nucleotide polymorphism of iNOS C150T was examined for 454 Japanese health checkup examinees (126 males and 328 females) aged 35 to 85 years without a history of cancer and 202 gastric cancer patients (134 males and 68 females) aged 33 to 94 years with pathologically confirmed diagnosis of gastric adenocarcinoma.

RESULTS: The iNOS C150T polymorphism was not associated with gastric atrophy or with *H pylori* seropositivity. The odds ratio (OR) of the C/T + T/T for gastric cancer was increased without statistical significance (OR=1.19, 95% confidence interval (CI): 0.68-2.08). In the differentiated subgroup ($n = 113$), however, the OR of the C/T genotype for gastric cancer was significant (OR = 2.02, 95% CI: 1.04-3.92) relative to the C/C genotype. In addition, considering the location of gastric cancer ($n = 105$), there were significant differences between the controls and non-cardia group with the OR of 2.13 (95% CI: 1.08-4.18) for C/T and 1.94 (95% CI: 1.00-3.78) for C/T + T/T.

CONCLUSION: The iNOS C150T polymorphism is associated with the risk of *H pylori*-related gastric cancer

INTRODUCTION

Gastric cancer is the second most frequent cancer in the world, accounting for a large proportion of cancer cases in Asia, Latin America, and some countries in Europe^[1]. *H pylori* strains carrying the cytotoxin-associated gene A (*cagA*) are strongly associated with increased risk of gastric adenocarcinoma^[2]. However, only some of those infected with *H pylori* developed *H pylori*-related gastric cancer, even in Asian countries including Japan with high prevalence of *cagA*-positive *H pylori* infection. Therefore, it is important to examine any host genetic predisposition to *H pylori*-related gastric cancers.

Nitric oxide (NO) produced by activated phagocytes has been reported to play a role in the processing of carcinogenesis^[3-5]. NO is synthesized enzymatically from L-arginine by a family of three distinct nitric oxide synthase (NOS) isoforms^[5,6]. Two NOS isoforms, eNOS (expressed in vascular endothelial cells) and nNOS (expressed in neurons of the central and peripheral nervous system), are constitutively expressed and are calcium dependent. The third isoform, inducible NOS (iNOS), is calcium independent, and expressed in response to bacterial endotoxins and cytokines to cause sustained NO release. iNOS is one of the most important enzymes involved in the pathway of reactive oxygen and nitrogen species metabolism in the presence of *H pylori* infection. iNOS can produce larger amounts of NO than either eNOS or nNOS. iNOS expression in the gastric mucosa is higher in *H pylori* positive gastric cancer patients than in

H. pylori negative patients^[7].

Several types of polymorphisms have been identified in the promoter region of the iNOS gene: G to C at -954, C to T at -1173, and tandem repeat number polymorphism of (TAAA)_n and (CCTT)_n^[8-11]. iNOS production is mainly regulated at the transcriptional level^[12]. The human iNOS gene comprises 27 exons, with the transcription start site in exon 2 (E2) and the stop codon in E27^[13]. Johannesen *et al*^[14] have detected 10 polymorphisms in 8 exons of the iNOS gene, of which one polymorphism (C150T) in exon 16 resulting in an amino acid substitute, Ser608Leu, showed the only distorted transmission in the transmission disequilibrium test. This polymorphism is associated with cigarette- and alcohol- induced gastric cancer in Chinese population^[15], indicating that iNOS Ser608Leu allele may have a dramatic effect on the enzyme activity.

Accordingly, we have investigated the associations of iNOS C150T polymorphism with *H. pylori* seropositivity, gastric atrophy and gastric cancer in Japanese population.

MATERIALS AND METHODS

Study subjects

Detailed information of the characteristics of healthy controls and gastric cancer patients in this study has been published in our previous paper^[16]. Briefly, the control group included 454 health checkup examinees (126 males and 328 females) aged 35 to 85 years with no history of cancer who attended a health checkup program supported by the Nagoya Municipal Government in August and September, 2000. The case group consisted of 202 patients (134 males and 68 females) aged 33 to 94 years with a pathologically confirmed diagnosis of gastric adenocarcinoma undergone tumor resection in different hospitals affiliated to Nagoya University. Informed consent was obtained from all subjects. Approval for the study was given by the relevant ethical committees.

Tests for *H. pylori* antibody and pepsinogens

Anti-*H. pylori* IgG antibody tests, high molecular weight campylobacter-associated protein (HM-CAP) ELISA (Enteric Products Inc., Westbury, NY) and HM-CAP with antigens extracted from clinically isolated Japanese *H. pylori* strain (J-HM-CAP) ELISA (Kyowa Medex, Tokyo, Japan), were used for the identification of *H. pylori*-infected participants. An ELISA value of 2.3 or over was regarded as positive for both tests. The infection was confirmed in all gastric cancer cases by culture and bacteriological tests (Gram-negative, oxidase, catalase, and urease test-positive spiral, curved rods) using biopsy specimens before gastric resection. Pepsinogens I and II (PG I and PG II) in serum were measured by radioimmunoassay using a commercially available kit (DINABOT, Tokyo, Japan). Gastric atrophy was defined as PG I < 70 ng/mL and PG I/PG II ratio < 3. These parameters for atrophy are widely used in Japan and have been validated against histological confirmatory studies.

Genotyping

DNA was extracted from the buffy coat fraction by Qiagen QIAamp DNA blood mini kit (QIAGEN

Table 1 The sex-age-adjusted ORs and 95% CIs of the iNOS C150T genotypes for gastric atrophy (GA) among *H. pylori* seropositive controls

iNOS polymorphism	n	GA n (%)	OR	95% CI
C/C	217	121 (55.8)	1.00	Reference
C/T	30	16 (53.3)	0.90	0.42-1.94
T/T	3	0 (0)	0	-
C/T+T/T	33	16 (48.5)	0.75	0.36-1.56

Table 2 The sex-age-adjusted ORs and 95% CIs of the iNOS C150T genotypes for gastric cancer

iNOS polymorphism	Cases ¹ n (%) (n = 201)	Controls n (%) (n = 454)	OR	95% CI
C/C	175 (87.1)	403 (88.8)	1.00	Reference
C/T	25 (12.4)	48 (10.6)	1.22	0.69-2.17
T/T	1 (0.50)	3 (0.66)	0.71	0.07-7.54
C/T + T/T	26 (12.9)	51 (11.2)	1.19	0.68-2.08

¹One case subject could not be genotyped.

Inc., Valencia, CA). We carried out PCR-RFLP to identify iNOS C150T gene genotype as previously described by Shen *et al*^[15]. However, the way to do PCR was somewhat different. Genomic DNA was used per 25 µL of reaction with 0.12 mmol/L dNTPs, 25 pmol of each primer, 0.5 units of "Ampli-Taq Gold", and 2.5 µL GeneAmp 10 × PCR buffer including 15 mmol/L MgCl₂ (Perkin-Elmer Corp., Foster City, CA). Amplification conditions were 10 min of initial denaturation at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 58°C and 1 min at 72°C, then a final extension at 72°C for 5 min. The PCR product was digested with a restriction enzyme (*Tsp* 509 I) by the same way as previously described by Shen *et al*^[15].

Statistical analysis

The strength of associations of *H. pylori* seropositivity, gastric atrophy and gastric cancer with the iNOS C150T polymorphisms was measured as odds ratios (ORs). ORs adjusted for sex and age with 95% confidence intervals (CIs) were calculated using logistic regression analysis. Hardy-Weinberg equilibrium was tested for iNOS C150T polymorphism. We used two-sided *P* values. *P* < 0.05 was considered statistically significant. These calculations were performed by computer program STATA Version 8 (STATA Corp., College Station, TX).

RESULTS

The characteristics of study subjects have been described elsewhere^[16]. Only one case could not be genotyped. The distributions of the iNOS C150T gene was in the Hardy-Weinberg equilibrium ($\chi^2 = 1.37$ and *P* = 0.24). The iNOS C150T polymorphism had no significant effect on *H. pylori* seropositivity. Table 1 shows the sex-age-adjusted ORs and 95% CIs of the iNOS C150T genotypes for gastric atrophy among *H. pylori* seropositive controls. The iNOS C150T polymorphism was not associated with gastric

Table 3 The distribution of the *iNOS* C150T genotype in case, considering the location of gastric cancer and the phenotype *iNOS*

Polymorphism	Phenotype ¹	Total cases ² <i>n</i> (%) (<i>n</i> = 185)	OR	95% CI	Non-cardia <i>n</i> (%) (<i>n</i> = 177)	OR	95% CI	Cardia <i>n</i> (%) (<i>n</i> = 8)	OR	95% CI
C/C	Differentiated	93 (82.3)	1.00		86 (81.9)	1.00		7 (87.5)	1.00	
C/T	Differentiated	20 (17.7)	2.02	1.04-3.92 ^a	19 (18.1)	2.13	1.08-4.18 ^a	1 (12.5)	1.28	0.15-11.0
T/T	Differentiated	0 (0)	0		0 (0)	0		0 (0)	0	
C/T + T/T	Differentiated	20 (17.7)	1.84	0.96-3.54	19 (18.1)	1.94	1.00-3.78 ^a	1 (12.5)	1.16	0.14-9.95
C/C	Undifferentiated	69 (95.8)	1.00		69 (95.8)	1.00		0	-	
C/T	Undifferentiated	2 (2.78)	0.25	0.06-1.06	2 (2.78)	0.25	0.06-1.06	0	-	
T/T	Undifferentiated	1 (1.39)	1.74	0.17-18.1	1 (1.39)	1.74	0.17-18.1	0	-	
C/T + T/T	Undifferentiated	3 (4.17)	0.35	0.10-1.17	3 (4.17)	0.35	0.10-1.17	0	-	

^a $P < 0.05$ vs control group. ¹Information on gastric cancer phenotype was not available for 16 cases; ²One case subject with undifferentiated type could not be genotyped.

atrophy. Table 2 shows the sex-age-adjusted ORs and 95% CIs of the *iNOS* genotypes for gastric cancer. The OR of the C/T+T/T genotype for gastric cancer was increased without significance.

In the same way as our previous report^[17], we divided case subjects into differentiated and undifferentiated type according to their tumor phenotypes, referring to Nakamura *et al.*^[18] (Table 3). We could not get information on the phenotypes for 16 cases. In the differentiated type subgroup, the OR of the C/T genotype for gastric cancer was significant (OR = 2.02, 95% CI: 1.04-3.92). Considering the location of gastric cancer, there were significant differences between the controls and non-cardia group with the OR of 2.13 (95% CI: 1.08-4.18) for C/T genotype and 1.94 (95% CI: 1.00-3.78) for C/T+T/T genotype. The prevalence of *H. pylori* seropositivity in the cases and healthy controls was 100% and 55.1%, respectively. All non-cardia subgroups with differentiated phenotype had gastric atrophy. On the other hand, atrophy was present in 34.8% of 454 controls, 21 of which were seronegative.

Especially compared with *H. pylori* seropositive controls or gastric atrophy positive controls, the corresponding ORs were 2.20 (95% CI: 1.08-4.49) for C/T genotype and 1.94 (95% CI: 0.97-3.89) for C/T+T/T genotype.

DISCUSSION

This study showed that the *iNOS* C150T polymorphism was associated with gastric cancer where the cell type was differentiated type and located in non-cardia, namely *H. pylori*-related gastric cancer. Our control *iNOS* gene frequency is coincident with the results presented by Shen *et al.*^[15], which represents the genotype frequency in China.

We could not evaluate the interaction between *iNOS* C150T polymorphism and smoking-induced risk of gastric cancer as the study of Shen *et al.*^[15], because we have no information on smoking habit in case group. *iNOS* is expressed in response to bacterial endotoxins and cytokines to cause sustained NO release. This excess NO would contribute to the development of gastric atrophy, reacting with superoxide produced by *H. pylori* infection^[19] to form

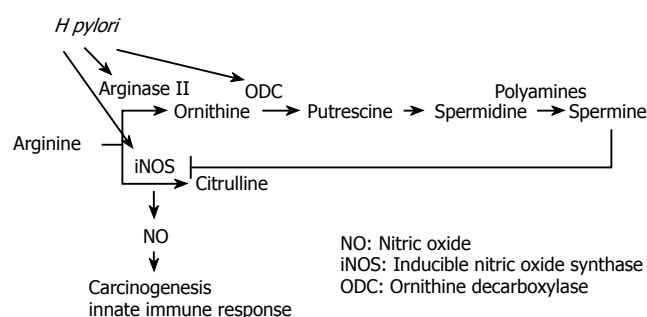


Figure 1 Regulation of Nitric oxide (NO) and polyamine pathways. *H. pylori* induces inducible nitric oxide synthase (*iNOS*) expression, arginase II and ornithine decarboxylase (ODC). *iNOS* competes with arginase for arginine. Gastric cancer relates to the high expression of *iNOS*. *H. pylori*-induced *iNOS* is inhibited by spermine produced by the arginase-ODC pathway.

peroxynitrite which has strong oxidizing properties. We chose the seropositive controls as the comparison group. The *iNOS* C150T polymorphism, however, was not associated with gastric atrophy as the precursor lesion of gastric cancer in this study.

It was reported that immune dysregulation induced by *H. pylori* in which stimulated spermine synthesis by the arginase-ornithine decarboxylase (ODC) pathway inhibits *iNOS* translation and NO production, can lead to persistence of the bacterium^[20] (Figure 1). We hypothesized that the *iNOS* C150T polymorphism might affect *H. pylori* infection. But, this gene polymorphism was not associated with *H. pylori* seropositivity in this study, confirming the finding that there is no correlation between *H. pylori* infection and *iNOS* expression^[21]. On the contrary, it has been reported that *iNOS* detection is significantly associated with *H. pylori* infection^[22,23].

There are many reports concerning the high expression of *iNOS* in gastric cancer, which increases with the stage of cancer and lymph node metastasis^[24-26]. It was reported that the long forms of *iNOS* promoter region are associated with intestinal gastric cancer in Japanese women^[27]. Nam *et al.*^[28] reported that *iNOS* contributes to *H. pylori*-associated gastric carcinogenesis, and that *H. pylori* is associated with non-cardiac tumor but not with cardiac tumor^[29]. The

high incidence of *H pylori* infection in patients with gastric cancer including both intestinal type and diffuse type, particularly in those with intestinal type has been confirmed^[30]. Our study has confirmed that iNOS C150T polymorphism is related to the risk of *H pylori*-related gastric cancer, by stratification of iNOS genotype frequency among cases according to the location of gastric cancer and histologic phenotype. It may take decades for superficial gastritis to progress to atrophic gastritis. *H pylori* infection is highly associated with each stage of this progression^[31]. Loss of serological markers of *H pylori* infection following onset of severe atrophy and intestinal metaplasia is a well described phenomenon, including in Japan^[32]. Considering these facts, the comparison with the controls with *H pylori* seropositivity and/or gastric atrophy might be adequate to evaluate the effect of the genotype on *H pylori*-related gastric cancer risk. In fact, the corresponding OR was significantly stable (OR = 2.20 for C/T, 95% CI: 1.08-4.49) in our study.

iNOS differs independently of Ca²⁺ from the constitutive forms of NOS (eNOS and nNOS). One major divergence in the close sequence similarity is a 40-50-amino acid insert in the middle of the FMN-binding domain of eNOS and nNOS. When this insert is removed from nNOS, the deleted mutant retains maximal NO synthesis activity at a lower concentration of free Ca²⁺ than the wild type enzyme^[33]. The iNOS C150T polymorphism is located at the position near to this deletion. The amino acid change in E16 may be of functional interest. As in the study of Shen *et al*^[15], our study indicated that those with T allele might increase iNOS expression and the level of NO in gastric mucosa. But specific functional tests of this polymorphism remain to be elucidated.

It is important to discuss our finding that iNOS C150T polymorphism is associated with *H pylori*-related gastric cancer but not with *H pylori* infection or with gastric atrophy (especially the association with the latter two is controversial). iNOS-derived NO is a central effector molecule in the innate immune response to pathogens, with essential antimicrobial functions in host defense. *H pylori* induces both arginase II and ODC in macrophages^[34] (Figure 1). Arginase converts L-arginine to L-ornithine, which is metabolized by ODC to produce putrescine that is converted to polyamines (spermidine and spermine). Spermine inhibits *H pylori*-stimulated NO production in macrophages by a post-transcriptional effect on iNOS translation^[20]. The temporal switch of arginine as a substrate for the cytostatic iNOS/NO axis to the pro-growth arginase/ornithine/polyamine and proline axis is regulated by inflammatory cytokines as well as interregulated by the arginine metabolites themselves. Satriano^[35] has proposed that agmatine, converted from arginine by arginine decarboxylase, coordinates the early and repair phase pathways of arginine in the inflammatory response as a gating mechanism at the transition from the iNOS/NO axis to the arginase/ODC/polyamine axis. Therefore the stage of *H pylori* infection and gastric atrophy might follow the arginase/ODC/polyamine axis. On the other hand, the stage of gastric cancer might follow the iNOS/NO axis. In addition, there is

no significant association between cagA positive *H pylori* strains and iNOS expression^[20]. CagA might affect the mechanisms that regulate this temporal switch, resulting in the arginase/ODC/polyamine axis.

In conclusion, iNOS C150T polymorphism is associated with the risk of *H pylori*-related gastric cancer in Japanese and Chinese population. This polymorphism may play an important role in increasing the risk of gastric cancer in Asian countries with the highest rates of gastric cancer. The confirmation of this finding requires much larger studies of different ethnic groups to stimulate the interest in the molecular mechanisms of this polymorphism function.

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RAPID COMMUNICATION

Randomized clinical study of five days apostrophe therapy with mebendazole compared to quinacrine in the treatment of symptomatic giardiasis in children

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Abstract

AIM: To compare the efficacy and safety of five days apostrophe therapy of mebendazole (MBZ) versus quinacrine (QC) on human giardiasis in children.

METHODS: A clinical trial was carried out in paediatric patients (aged 5-15 years) with confirmed symptomatic *G. duodenalis* mono-infection. Patients were randomly assigned to receive either MBZ [200 mg taken three times per day (TID) ($n = 61$)] or QC [2 mg/kg bodyweight tid ($n = 61$)], both for five days. Follow-up faecal samples were obtained at 3, 5 and 7 d after the end of the treatment.

RESULTS: Although the frequency of cure was higher for QC (83.6%) than for MBZ (78.7%), the difference was not statistically significant ($P > 0.05$). Adverse events were reported more in the QC group ($P < 0.05$), all of them transient and self-limiting.

CONCLUSION: Despite final cure rates occurring lower than expected, the overall results of this study reconfirmed the efficacy of MBZ in giardiasis and also indicate that, although comparable to QC, at least in this setting the 5 d course of MBZ did not appear to improve the cure rates in this intestinal parasitic infection.

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Key words: *Giardia* infection/drug therapy; Children; Mebendazole/therapeutic use; Quinacrine/therapeutic use; Benzimidazoles/therapeutic use; Giardiasis/drug therapy; Cuba

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INTRODUCTION

Giardiasis is one of the commonest intestinal parasitic diseases diagnosed worldwide. Its clinical presentation is highly variable, most of the time it is characterized by mild and self-limiting signs and symptoms. However, it is not unusual that in some cases, this disease can become a significant cause of morbidity, resulting in malabsorption of fats, vitamins and lactose with serious consequences, particularly in children, causing failure-to-thrive syndrome^[1,2].

Classically, 5-nitroimidazole compounds have been accepted for decades the world over as the "gold standard" for treatment of patients with giardiasis. However, there is an increasing number of treatment failures reported in the literature, requiring repeated courses of the same drug, change to other compounds or make a combination of two anti-*Giardia* drugs for therapy^[3,4]. Consequently, newer and older drugs, such as nitazoxanide^[5,6] and chloroquine^[7] respectively, have been proposed as alternatives for the treatment of this intestinal infection.

In recent years, chemotherapy with mebendazole (MBZ), the benzimidazole-carbamate compound that has been extensively used in the treatment of helminthic infections, has also demonstrated *in vitro* activity against *Giardia duodenalis* (*G. duodenalis*)^[8-14], the causative agent of human giardiasis. Clinical experience has also shown therapeutic benefits for the use of MBZ in this infection^[15-17], although it is usually accepted, the optimal dosage and duration of the treatment remain to be determined. For this reason, in the present study it was decided to evaluate the efficacy and safety of a five-day regimen of MBZ versus quinacrine (QC) in the treatment of giardiasis in a group of Cuban paediatric patients.

MATERIALS AND METHODS

Patient selection

This study was carried out at the Gastroenterology in-

stitute in Havana, Cuba. In order to recruit a sufficient number of patients for this trial, all the specialists of the institution were invited to refer their paediatric patients (aged 5-15 years) who were received seeking treatment for symptomatic acute *G. duodenalis* infection with or without diarrhoea from May to December, 2003. To be eligible for the study a child had to have mono-infection with *G. duodenalis* (proven by microscopic examination of faecal samples, as wet mounts and/or after Ritchie concentration^[18]). Patients were excluded from the study if any of the following conditions were present: (1) known history of sensitivity to any of benzimidazole compounds or QC, (2) had received any antiparasitic chemotherapy in the preceding 4 wk, (3) diseases other than giardiasis. Also were excluded those who were unlikely to attend follow-up examinations.

The protocol was approved by the Research and Ethics committee of the institute. Parents or legal guardians of each child were fully informed about the aim of the study, the characteristics of the drugs under investigation and they were told that his/her child's participation was optional. Written informed consent was obtained from them prior to trial enrollment.

The sample size for each treatment group (n) needed to ensure sufficient statistical power (80%) to reject the null hypothesis that MBZ and QC are not equally effective (in terms of a parasitological cure) with a significant level of 5%, was calculated according to Armitage and Berry^[19]. The following equation was used:

$$n = \left(\frac{Z_{2\alpha} \sqrt{2p(1-p)} + Z_{2\beta} \sqrt{p(1-p_1) + p_2(1-p_2)}}{p_1 - p_2} \right)^2$$

where:

π_1 : denotes the proportion of population cured with standard treatment.

π_2 : denotes the proportion of population cured with the assayed treatment.

$Z_{2\alpha} = 1.96$

$Z_{2\beta} = 0.842$

One hundred and twenty two children were required (i.e. 61 in each treatment arm).

Experimental design

Patients who (a) were eligible, (b) met none of the exclusion criteria, and (c) one of their parents or legal guardians had given written informed consent for the trial, were included in the study. A random-number table was used to allocate each of these 122 children to receive either MBZ (Reynaldo Gutierrez Pharmaceutical, Havana) 200 mg thrice a day or QC (Reynaldo Gutierrez Pharmaceutical, Havana) at 2 mg/kg bodyweight thrice daily, both for 5 d. The drugs were provided at no charge.

A detailed history was taken from the accompanying parent or legal guardian; a standardized questionnaire was used to record clinical signs and symptoms before starting treatment and at the end. Also, a physical examination was carried out, and each child was weighed.

Comprehensive oral instructions were given to all children and parents or legal guardians accompanying them

Table 1 Demographic characteristics of the study patients randomized to MBZ and QC at admission

	MBZ group <i>n</i> = 61	QC group <i>n</i> = 61
Sex		
Male: <i>n</i> (%)	28 (45.9)	26 (42.6)
Female: <i>n</i> (%)	33 (54.1)	35 (57.3)
Age (yr)		
Mean (range)	8.2 (5-15)	9.7 (5-15)

in an attempt to maintain a high level of compliance with the study plan, including the administration of the drug and the importance of good hygiene and of measures they could take to reduce the risk of giardial infection in the future.

Follow-up

The evaluation of efficacy of the chemotherapy was based on parasitological response to therapy assessed by the same laboratory tests that were done initially. Parents or legal guardians of each child were asked to provide three faecal samples on d 3, 5, and 7 after treatment completion in order to avoid including the possibility of re-infection, considering it is a very frequent phenomenon in children, mainly in developing countries. Also, they were encouraged to return to the clinic at any time, if they considered that his or her child was ill. A child was only considered to be cured, if no *Giardia* trophozoites or cysts could be found in any of the three post-treatment faecal specimens.

Evaluation of adverse events

Irrespective of their causal relationship to study treatment, details of all clinical adverse events reported spontaneously at any time during the trial, regardless of their relationship to the study drugs and those elicited by the investigators with no leading questions in each of the clinic visits scheduled, were taken into account. Adverse event was defined as the development of any sign or symptoms that did not exist before or which became more serious following the commencement of the treatment. Serious adverse events were defined as death, any life-threatening, disabling or incapacitating events, or those requiring hospitalization.

Statistical analysis

The data on the parasitological response and adverse events were taken on pre-designed record forms and subsequently analysed to determine the frequency of each response/effect. The statistical significance of differences between mean values was determined using the Student's *t*-test. Where appropriate Fisher exact test (χ^2 test) was used to establish the significance of differences in proportions.

RESULTS

All 122 paediatric patients who began treatment completed this study and were included in the statistical analysis. Characteristics of the treatment groups at the entry with

Table 2 Cure rates and drug-related adverse events reported by treatment groups

	MBZ group <i>n</i> = 61	QC group <i>n</i> = 61
Cure rate	48 (78.7%)	51 (83.6%)
Any adverse event	14 (22.9%)	36 (59%)
Abdominal pain	11 (18 %)	10 (16.3%)
Vomiting	3 (4.9%)	14 (22.9%)
Nausea	3 (4.9%)	14 (22.9%)
Headache	1 (1.6%)	11 (18%)
Yellowish coloration of the skin	-	15 (24.6%)

regard to sex and age are presented (Table 1). There were no significant-between the groups- differences in demographics ($P > 0.05$).

The efficacy did not differ significantly between the two groups ($P > 0.05$), although it was slightly lower in the MBZ group [48 out of 61 (78.7%)] than that seen in the group treated with QC (83.6%) (Table 2).

Adverse events recorded with any treatment are also shown in Table 2. The therapy with the drugs was well tolerated and did not produce adverse events that warranted discontinuation of the study medication; in fact, no patients had to stop treatment because of potentially drug-related adverse events. Of the 122 patients studied, 72 (59%) had no adverse events, but 50 [14 in the MBZ group (22.9%) and 36 (59%) in the group treated with QC] reported at least one such event, none of them unexpected. Nausea, vomiting, headache and yellow discoloration were reported statistically significant by the QC group ($P < 0.05$). All adverse events were graded as mild, transient in nature, and did not require administration of drugs for relief or hospitalization. Except for yellow discoloration which lasted longer, the rest of the clinical adverse events usually resolved within 1-2 d and any of them were severe enough to interfere with activities of daily living.

DISCUSSION

Treatment failures with the currently available drugs used to treat giardiasis have provided a continuous stimulus to search for other therapeutic alternatives. MBZ has been one of the drugs proposed in the treatment of this infection due to the observations of Hutchison *et al*^[20] who during their investigation of the activity of this drug against intestinal nematodes realized that some cases of *Giardia* infection could also be cured. Lately, although *in vitro* studies not only confirmed the effect of MBZ on *Giardia* trophozoites, but have also served to clarify its activity against this protozoan, a number of clinical studies have been performed in adults and children comparing this drug with the currently available anti-giardial drugs. This could be, in part, because giardiasis as a new indication of MBZ is still a topic of discussion due to the contradictory clinical reports concerning its efficacy. Thus, while most of the published clinical data documented the effect of this drug in *Giardia* infections, in contrast there are some reports that show the failure of this drug to clear their

patients' symptoms or stop the patients from excreting *Giardia* cysts using MBZ for 1 and 5 d, respectively^[21,22], and interestingly, Rousham^[23] noticed that during a deworming study in northern Bangladesh, the prevalence of *G. duodenalis* increased significantly among children receiving periodic treatment with MBZ; for this reason there is a continuous necessity to evaluate treatment dosage and regimens with this drug.

The present study again confirms previous findings that MBZ is an attractive and efficacious agent against *Giardia* infections because it can be easily administered and has not been associated with serious adverse events. In a previous trial of MBZ, Bulut *et al*^[24] used this drug at 100 mg thrice for 1 d and for 7 d and obtained responses of 41.7% and 58.3%, respectively. Other studies have reported higher parasitological response rates e.g., Rodriguez-Garcia *et al*^[5], found that treatment with administered 100 mg of MBZ every 12 h, for 3 d achieved a cure rate of 80.4% that was comparable to 100 mg of nitazoxanide administered in the same way. In a closely related trial, similar outcomes were obtained by Sadjjadi *et al*^[16], who used 200 mg of MBZ thrice daily for 5 d -which resulted in somewhat higher cure rates [43 out of 50 (86%)] than that seen in the present study- and found a frequency comparable to that obtained by using a 7-d course of metronidazole.

Based on the experience of Sadjjadi *et al*^[16] and on a previous study carried out in Cuba in paediatric patients for 3 d where the cure rates obtained were 78.1%^[17], we decided to treat patients in this current study with a 5-d course of MBZ at 200 mg thrice a day in order to see if the response rate could be improved after this prolonged course; however, no additional benefit in the efficacy rate could be demonstrated.

Contrary to what was expected, it was noted that QC resulted in considerably lower cure rates than that which have been previously reported by other authors. This drug was widely used for the chemosuppression of malaria and used to be the front line drug for the treatment of adult giardiasis, but it was superseded for these purposes by chloroquine and metronidazole, respectively. QC has been regarded the most efficacious drug of any of the anti-*Giardia* therapeutics by some researchers^[25] because its clinical efficacy in giardiasis has been found over 90%. This drug also has the benefits that even in patients with severe diarrhoea its intestinal absorption is not interfered^[26] with and it has the additional epidemiological advantage that it could also kill cysts^[27]. However, this drug has the potential of adverse events, including yellow discoloration of the skin, bitter taste, nausea and vomiting, and it has also been reported in toxic psychosis^[28]. For these reasons, at this moment this old drug might experience a revival in the clinical management of patients where other drugs have failed (to whom it has almost wholly been reserved). Nevertheless, clinical reports in which *Giardia* has not been eliminated despite one or more appropriate courses with QC have been published^[3,29]. Additionally, in one study resistance against QC could be induced in *Giardia* laboratory stocks^[30].

In the present study, no new or previously undescribed adverse events occurred. Both drugs were well tolerated,

resulting in good patient compliance. In no case, adverse events observed lead to discontinuation of the drugs. The adverse events observed were all mild, transient in nature and self-limiting; all generally occurred at similar frequencies to those observed in previous trials with the same drugs. In general, MBZ is very well tolerated which could be due, in part, to the advantage of being hardly absorbed from the gut (no more than 20% of the dose, even after a fat rich meal)^[31].

One possible weakness in the current study was that for practical reasons it was conducted in an open fashion. As the two drug treatments look very different and the number of tablets to take daily varied it was impossible to make the study blind. Certainly, in the market it would have had been possible to obtain placebos for the two drugs but this would have been costly for the study. This could be a limitation and consequently, despite well-defined pre-study criteria for evaluating efficacy and safety, evaluation of the treatment response and possible cause of adverse events could have been somewhat biased; but it could not have influenced the major result (eradication of *Giardia* infection) because the efficacy analysis was done by the laboratory department where those checking post-treatment faecal samples were unaware of the treatment allocation and had no clinical involvement with the paediatric patients or their parents.

For us, this study has the strength that in many countries MBZ is not considered even a major alternative treatment for giardiasis; therefore, it is useful information that there is a therapeutically effective high dose used daily at least three times the usual dose for helminthic infections caused by hookworms and six times that used for enterobiasis. Additionally, there have been several studies of MBZ for giardiasis, but it is the first to compare with QC which has permitted evaluation and update of the current efficacy of QC in Cuban children with giardiasis.

Where does the present data lead us with the use of MBZ in giardiasis? That is the question that could have been asked to answer given the current context. We shall approach the problem by offering a series of related conclusions: clearly, our study adds support to the observation that this drug has a place as an alternative treatment of giardiasis in children when (a) other first-line drugs have failed, (b) in areas where this infection and other sensitive organisms, e.g., intestinal nematodes, are prevalent, (c) in patients with a history of known sensitivity to any of the currently anti-giardial compounds, and (d) possibly, as adjunctive therapy in combination with other available anti-giardial drugs in order to offer potentially higher cure rates.

Taking into account that the ideal antiparasitic agents would be efficacious, easily dosed and administered, inexpensive, and with few adverse effects, in view of the above-given data for MBZ and QC, neither would be first line drugs, but this paper may help to inform about choices for those people where other currently recommended drugs have failed. MBZ belongs to a chemical family that differs from 5-nitroimidazoles, acts against *Giardia duodenalis* by a different mechanism, and has a different safety profile. In the present study it was at least as effective as, and better tolerated than QC. All of these advantages

should not be forgotten when we are treating giardiasis, but the benefits of extending treatment from three to five consecutive days were not apparent, at least in this setting.

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Diagnostic dilemma of abdominal tuberculosis in non-HIV patients: An ongoing challenge for physicians

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Abstract

AIM: To assess the clinical features, yield of the diagnostic tests and outcome of abdominal tuberculosis in non-HIV patients.

METHODS: Adult patients with discharge diagnosis of abdominal tuberculosis (based upon; positive microbiology, histo-pathology, imaging or response to trial of anti TB drugs) during the period 1999 to 2004 were analyzed. Patient's characteristics, laboratory investigations, radiological, endoscopic and surgical findings were evaluated. Abdominal site involved (intestinal, peritoneal, visceral, and nodal) and response to treatment was also noted.

RESULTS: There were 209 patients enrolled. One hundred and twenty-three (59%) were females. Symptoms were abdominal pain 194 (93%), fever 134 (64%), night sweats 99 (48%), weight loss 98 (47%), vomiting 75 (36%), ascites 74 (35%), constipation 64 (31%), and diarrhea 25 (12%). Sub-acute and acute intestinal obstruction was seen in 28 (13%) and 12 (11%) respectively. Radiological evidence of pulmonary tuberculosis was found in 134 (64%) patients. Basis of diagnosis of abdominal tuberculosis were radiology (Chest and barium X-Rays, Ultrasound and CT scan abdomen) in 111 (53%) and histo-pathology (tissue obtained during surgery, colonoscopy, CT or ultrasound guided biopsy, laparoscopy and upper gastro intestinal endoscopy) in 87 (42%) patients. Mycobacterium culture was positive in 6/87 (7%) patients and response to therapeutic trial of anti tubercular drugs was the basis of diagnosis in 5 (2.3%) patients. Predominant site of involvement by abdominal TB was intestinal in 103 (49%) patients, peritoneal in 87 (42%) patients, solid viscera in 10 (5%) and nodal in 9 (4%) patients. Response to medical treatment was found in 158 (76%) patients and

additionally 35 (17%) patients also underwent surgery. In a 425 ± 120 d follow-up period 12 patients died (eight post operative) and no case of relapse was noted.

CONCLUSION: Abdominal TB has diverse and non-specific symptomatology. No single test is adequate for diagnosis of abdominal tuberculosis in all patients. Abdominal TB in non-HIV patients remains an ongoing diagnostic dilemma requiring a high index of clinical suspicion.

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Key words: Abdominal tuberculosis; Gastrointestinal tuberculosis; Tuberculosis; Diagnosis and abdominal tuberculosis

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INTRODUCTION

In developing countries, tuberculosis is associated with poverty, deprivation, overcrowding, illiteracy, and limited access to health care facilities. While in developed worlds, tuberculosis is commonly accompanied with HIV infection, ageing population or due to trans-global migration^[1,2]. Approximately one eighth of total TB cases are extra pulmonary^[3,4], of these abdominal tuberculosis (ATB) accounts for 11%-16%^[5,6]. In HIV positive patients the incidence of extra pulmonary TB is up to 50%^[1,6].

TB involves the abdomen as the primary disease from the reactivation of a dormant focus acquired somewhere in the past or as a secondary disease when infections spread to the abdomen via swallowed sputum, hematogenous or spread from an infected neighboring organ or ingestion of unpasteurized milk^[2,5]. Abdominal TB may be, enteric (intestine involved itself), peritoneal, nodal (lymph nodes involvement) and solid visceral TB like liver, spleen, kidney and pancreas or in any combination of these four varieties. Intestinal lesions may be ulcerating, hyperplastic or combined^[7,8].

Diagnosis of abdominal tuberculosis is difficult because of (1) vague and non-specific clinical features or (2) low yield of mycobacterium culture or smear. Moreover,

costly and invasive procedures are required for obtaining tissue for histo-pathological examination or culture and these are not easily available in developing countries^[6,9]. Investigations like Imaging (ultrasound, barium X-Rays, and CT scan) and Mantoux test have only supportive value. In some cases, response to therapeutic trials of anti-tuberculous drugs is the basis of diagnosis that may cause a delay in the diagnosis of other diseases which mimic abdominal tuberculosis e.g. Crohn's disease, abdominal lymphoma and malignancy of abdominal organs^[5]. Therefore, diagnosis of abdominal tuberculosis is an ongoing challenge to the physicians, especially with limited resources.

The aims of this study were to examine the clinical features and distribution of the disease, diagnostic yield of various tests and outcomes of abdominal tuberculosis in non-HIV patients.

MATERIALS AND METHODS

Patients above 15 years of age with a diagnosis of abdominal TB on discharge during the study period January 1999 to December 2004 were retrieved using the International classification of diseases 9th revision with clinical modification (ICD-9-CM- USA). Diagnosis of abdominal TB was based upon (1) a positive Acid Fast Bacilli (AFB) smear or culture, (2) histo-pathology showing tubercular granuloma (with or without caseation), (3) radiological features compatible with tuberculosis on barium x-rays of the abdomen, ultrasound or CT scan of the abdomen, and (4) patients with a high index of clinical suspicion and negative diagnostic workup but showed a good response to therapeutic trial of anti TB medicines^[10].

Patient's demographic, clinical features associated with medical illnesses, family and past history of TB were evaluated. Laboratory tests, mantoux skin test, chest and abdominal imaging results, histo-pathology findings, Acid Fast Bacilli (AFB) staining and culture reports, ascetic fluid analysis, HIV screening, findings of upper gastrointestinal endoscopy, colonoscopy, laparoscopy or laparotomy were noted. Response to anti-tubercular treatment surgical procedure performed, clinical course and complications were also evaluated.

Statistical analysis

The Statistical package for social science (SPSS 11.5) IL-Chicago- USA standard version was used for data analyses. Descriptive analysis was done for demographic, clinical and radiographic features and results are presented as mean \pm SD and percentages for continuous variable and number and percentage for categorical variable. All *P*-values were two sided and considered as statistically significant if *P* < 0.05.

RESULTS

Demographics and clinical characteristics

A total of 209 patients were analyzed and 123 (59%) were female. Mean age was 33 ± 15 years. The most frequent symptoms were abdominal pain (194, 93%), fever (134, 64%), night sweats (99, 48%), weight loss (98, 47%), vomiting (75, 36%), ascites (74, 35%), constipation

Table 1 Characteristics of non-HIV patients with abdominal tuberculosis (*n* = 209) mean age 33 ± 15 years

Patients characteristic	<i>n</i>	%
Male	86	41
Female	123	59
Past History of TB	13	6
Family History of TB	6	3
Associated Pulmonary TB	10	5
Abdominal pain	194	93
Fever	134	64
Night sweats	99	48
Weight loss	98	47
Vomiting	75	36
Ascites	74	35
Constipation	64	31
Diarrhea	25	12
Sub acute intestinal Obstruction	28	13
Acute intestinal Obstruction	22	11

Results are presented as number (percentage).

Table 2 Diagnostic yield of various investigations in patients with abdominal tuberculosis

Investigation	<i>n</i> (Patients in which investigations performed)	Yield of diagnostic test <i>n</i> /%
Barium meal and follow through	70	58/83
Barium enema	34	15/44
Ultra sound	93	82/88
CT Scan Abdomen	35	28/80
Histopathology of surgical specimen	35	35/100
Histopathology of Colonoscopic biopsy	35	29/83
Histopathology of ultrasound and CT guided biopsy	28	14/50
Histopathology of Laparoscopic biopsy	5	5/100
Histopathology of upper GI endoscopic biopsy	10	4/40
AFB culture	87	6/7

Results are presented as number (percentage).

(64, 31%), and diarrhea (25, 12%). Sub acute and acute intestinal obstruction was present in 28 patients (13%) and 22 (11%) patients, respectively (Table 1). Average duration of symptoms before first presentation was 265 ± 150 d. Past history of treatment for tuberculosis was present in 13 (6.2%) patients and a family history of tuberculosis was found in 6 patients (2.9%). Important systemic diseases associated with abdominal TB were diabetes mellitus in 22 (10.5%) patients and cirrhosis of liver 15 (7.7%).

Basis of diagnosis and yield of various investigations in abdominal tuberculosis

Radiology: Out of 209 patients, 130 were subjected to abdominal radiological investigations and presumptive diagnosis of abdominal tuberculosis was made in 110 patients (53%) (Table 2) that included barium meal and follow through in 58/70 (83%), barium enema in

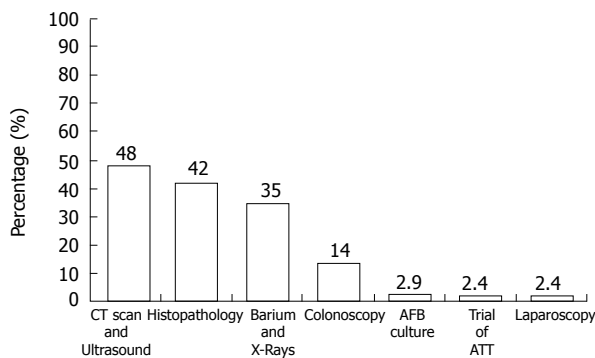


Figure 1 Basis of diagnosis in abdominal tuberculosis ($n = 209$).

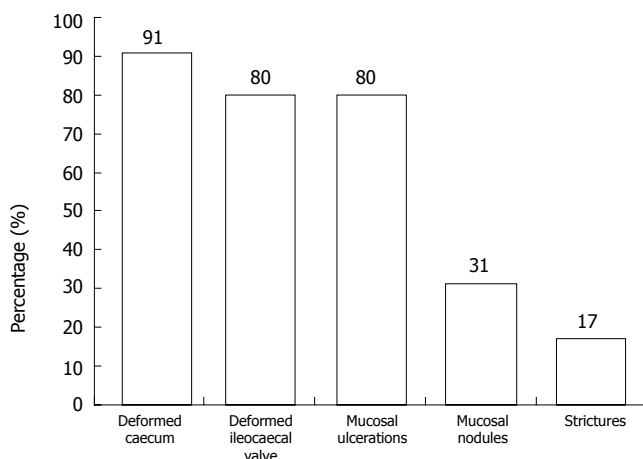


Figure 2 Colonoscopic findings in abdominal TB ($n = 35$).

15/34 (44%), ultrasound in 72/93 (77%) and CT scan abdomen in 28/35 (80%). Common features suggestive of abdominal tuberculosis on barium X-Rays were luminal narrowing with proximal dilation of bowel loops in 52/58 (90%) patients. Whereas on abdominal ultrasound and CT scan, findings suggestive of abdominal tuberculosis were; ascites 87/110 (79%), enlarged lymph nodes in 39/110 (35%), omental thickening 32/110 (29%) and bowel wall thickness in 28/110 (25%) patients. Features suggestive of pulmonary tuberculosis on x-rays chest were found in 134 (64%) patients and 10 (4.8%) patients had radiological features of active pulmonary TB (Figure1).

Histopathology: Histopathology was the basis of diagnosis in 87/113 patients. Diagnostic yield of histopathology on biopsy in abdominal TB was variable depending upon the method used to take the biopsy. Diagnostic yield of tuberculosis on surgical specimen were found in 35/35 (100%) patients. Biopsies obtained by colonoscopy had a yield of 29/35 (83%) and Ultrasound or CT guided biopsy of the viscera, omentum or LN suggestive of tuberculosis in 14/28 (50%) of patients. Diagnostic laparoscopic peritoneal biopsy was positive for abdominal tuberculosis in all (100%) patients. On the other hand biopsies taken during upper GI endoscopy favored diagnosis in 4/10 (40%) patients.

Common histological features on biopsy specimen was the presence of non caseating granuloma in 59/87

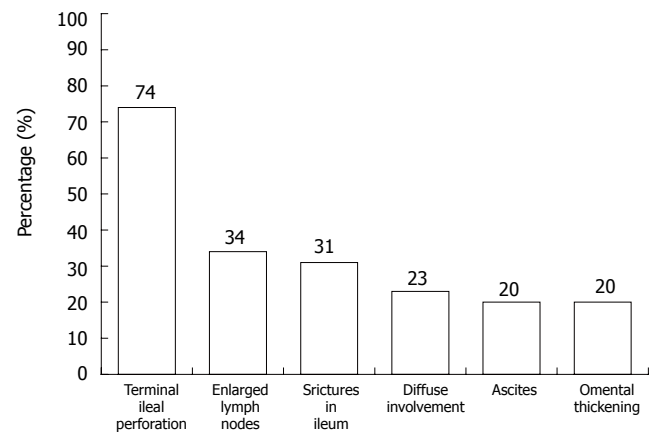


Figure 3 Laparotomy findings in abdominal TB ($n = 35$).

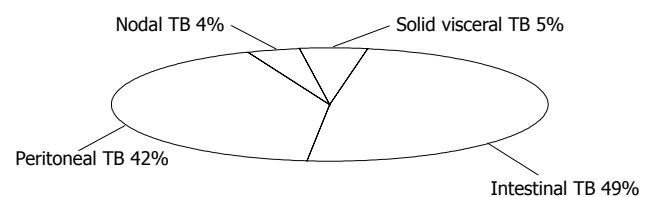


Figure 4 Predominant sites of involvement in abdominal TB ($n = 209$).

(68%) patients; however a central caseation was noted only in 22/87 (25%) cases and in 6/87 (7%) patients, chronic inflammatory cells infiltration but no definite granuloma was seen.

Samples (ascetic fluid, omental or intestinal biopsy tissues) were sent for acid-fast bacilli culture. The positive yield for acid-fast culture was only in 6/87 (7%) patients, all were on ascetic fluid.

In five (2.3%) patients with suggestive clinical history and negative diagnostic workup, response to therapeutic trial of anti TB drugs was the basis of diagnosis.

Colonoscopy and laparotomy findings in intestinal TB

Colonoscopic findings in 35 patients revealed deformed caecum in 32 (91%), irregular ileocaecal valve in 28 (80%), colonic mucosal ulcerations in 28 (80%), mucosal nodules in 11 (31%), and colonic strictures in 6 (17%) patients (Figure 2).

Laparotomy was performed in 35/209 (17%) of patients and notable findings were terminal ileal perforation in 26 patients, enlarged lymph nodes in 12, strictures in ileum in 11, diffuse involvement of the visceral and parietal peritoneum in 8, ascites in 7 and omental thickening in 7 patients (Figure 3).

Types of abdominal tuberculosis

Based upon predominant clinical features and investigations, site of abdominal TB involvement was intestinal in 103 (49%), peritoneal in 87 (42%), solid viscera in 10 (5%) and nodal in 9 (4%) patients (Figure 4).

Treatment and outcome

Antituberculous treatment was given to all patients.

158/209 (76%) patients responded to medical treatment alone and 35 (17%) patients with complications at admission required additional surgical intervention. Antituberculous treatment was comprised of isoniazid, rifampicin, ethambutol or streptomycin and pyrazinamide in various combination for a period of 9 to 12 mo. A follow-up of 425 ± 120 d was available in 197 (94%) patients. No patient showed a relapse of disease during this follow-up period. However three patients developed drug induced hepatitis. Two of them recovered with modification of drug therapy and one 42 year old female patient developed hepatic encephalopathy and died. In total 12 (6%) patients died due to various complications; eight of them were those who underwent surgery for the complications of abdominal tuberculosis.

DISCUSSION

Current series of abdominal tuberculosis patients without HIV have highlighted several important considerations. Female predominance was shown as one characteristic feature of abdominal tuberculosis in several studies in the past and also evident in our current series with females contributing to 59% of all patients. Possible reasons for female predominance apart from malnutrition, illiteracy, and poor access to health care facility may be contiguous spread from tuberculous salpingitis^[6,11,12]. In contrast, a study from India showed 69.4% patients with ATB were male^[3,13].

The most frequent symptom at presentation was abdominal pain, which was present in 93% of patients in our study. Mid abdominal colicky pain represented intermittent small bowel obstruction and was seen in 90%-100% of patients in other studies as well^[14]. This highlight the non-specific nature of abdominal pain and common feature that is present in abdominal malignancy and Crohn's disease^[6,12].

Radiological investigation is the mainstay in making presumptive diagnosis of abdominal TB^[12,15], this include chest x-rays, ultrasound or CT scan abdomen and barium studies. In this review, half of the patients (53%) had suggestive radiological findings on barium study, CT scan and or ultrasound abdomen. In another review, barium study was supportive of diagnosis of ATB in 60% of patients^[1]. However CT scan of the abdomen, which is a costly investigation, gives a better view of intestinal and extra intestinal structures^[16]. In our series 28 (13.4%) patients had CT scan of the abdomen with supportive features. Another interesting feature in the present series is the evidence of concomitant pulmonary tuberculosis on chest x-rays in 134 (64%) patients out of these 10 (4.8%) patients had features suggestive of active pulmonary TB. In the literature, associated active pulmonary TB is highly variable and is reported up to 29%^[3,17]. Diagnosis made on the basis of radiology is rapid, easy and less expensive but it is presumptive and cannot exclude completely other diseases like Crohn's disease and malignancies of solid abdominal viscera^[18].

Colon and terminal ileum are the most common site of involvement in intestinal variety of tuberculosis and in this regard colonoscopic images and biopsies are considered

to be a quick and good diagnostic tool. In the present series, colonoscopy was helpful in making diagnosis in 35 patients, out of them in 29/35 patients, colonoscopic biopsies were positive for presence of granuloma. Colonoscopy also helps in differentiating the colonic tuberculosis from Crohn's disease^[19]. In patients with intestinal TB on colonoscopy, ulcers, strictures, nodules, pseudo polyps, fibrous bands, fistulas, and deformed ileocaecal valve are seen. Mucosal ulcers in intestinal TB tend to be circumferential and are usually surrounded by inflamed mucosa. The ileocaecal valve is either patulous or destroyed in ATB and in few cases it is like fish mouth opening. On the other hand colonoscopic features that favor Crohn's disease such as aphthous ulcers with normal surrounding mucosa skipped lesions or the presence of cobble stoning^[19].

Laparoscopy and biopsy is safe and may help in diagnosing peritoneal TB because of increased peritoneal involvement. In different studies, laparoscopy was found helpful in the diagnosis up to 87%-92% of peritoneal tuberculosis^[20,21]. Peritoneal biopsy *via* mini-laparotomy should be considered if laparoscopy is non-diagnostic^[21,22].

Demonstrating tuberculous granuloma is probably the most important investigation for a definitive diagnosis of ATB. In our series of patients, histo-pathology was the basis of diagnosis in 87/209 patients; however a typical granuloma with caseation was found only in 22/87 of patients in our series. Moreover in our patients the yield of demonstrating tuberculous granuloma was high when the specimen was taken surgically or through laparoscopy but it is invasive, expensive and not easily available. On colonoscopic biopsies, if granuloma is non-caseating, interpretation is difficult because Crohn's disease cannot be excluded^[16].

Many authors advocated therapeutic trial with anti tubercular therapy but it should not be encouraged routinely as it may delay the diagnosis of malignancy, lymphoma and Crohn's disease^[1,23]. In the present series, 5 (2%) patients with suggestive history but negative workup, therapeutic trial of anti TB drugs therapy (ATT) was the basis of diagnosis. In the literature up to 40% patients were given therapeutic trial of anti TB drugs^[1].

Types of Abdominal TB

Intestinal TB was the predominant form in this cohort of patients and accounted for 103 (49%) of patients. The majority of patients in this review have ileocaecal involvement. This is in agreement with other reviews on abdominal tuberculosis in which intestinal type of abdominal tuberculosis was ranged from 50%-78%^[14]. In one study, jejunioileal and ileocaecal involvement was more than 75% of all gastrointestinal TB^[2]. Relatively common involvement of terminal ileum in intestinal tuberculosis is due to either physiological stasis, large surface area of this part of the intestine, complete digestion of food and abundant lymph nodes in the region^[9].

The other, commoner type of abdominal tuberculosis in our patients was peritoneal TB, seen in 87 (42%) patients. In the literature, peritoneal TB involvement is around 43%^[9]. Typically exudative fluid with predominant

lymphocytes is seen in ascetic fluid but efforts should be made to establish tissue diagnosis by peritoneal biopsy^[21].

Prognosis and outcome

Response to anti tubercular drugs is generally very good. In our series 158 (76%) patients responded to medical treatment alone and 35 (17%) patients who presented with complications at the time of admission required additional surgery. Other studies have also shown the similar frequency of surgical intervention in patients with abdominal tuberculosis that is around 20%-40%^[24].

None of our patients showed signs of relapse after 14 mo follow-up. Twelve patients in this series died; eight of them post operatively. A possible reason for increased mortality in operated patients may be due to late presentation and previous complications like malnutrition, perforation and sepsis.

Mortality due to abdominal tuberculosis ranged from 8 to 50 percent in various series. Advanced age, delay in initiating therapy, and underlying cirrhosis have been associated with higher mortality rates^[13,22].

Limitations of the study

One of the limitations in this study is the retrospective nature of the data set but it doesn't interfere with the objective of the study. Secondly, all tests were not done in every patient because each test is not indicated in every case. The other limitation was the inability to confirm diagnosis of abdominal tuberculosis either by culture of Acid Fast Bacilli or by PCR in all cases. Yield of AFB culture on biopsy specimen and ascetic fluid is low^[1]. Polymerase Chain Reaction is not yet standardized in diagnosis of tuberculosis^[25].

In conclusion, abdominal TB is a complex disease and has diverse symptomatology that is non-specific. Tissue diagnosis is mandatory for appropriate management but it is invasive, expensive and unfortunately not always conclusive either. A high index of clinical suspicion is required along with the help of multiple adjuvant diagnostic tools for diagnosis of ATB. Until the time when we have a specific test for diagnosis of abdominal tuberculosis, this remains a challenge for physician.

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RAPID COMMUNICATION

Dynamic expression of extracellular signal-regulated kinase in rat liver tissue during hepatic fibrogenesis

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Abstract

AIM: To investigate whether extracellular signal-regulated kinase 1 (ERK₁) is activated and associated with hepatic stellate cell (HSC) proliferation in fibrotic rat liver tissue.

METHODS: Rat hepatic fibrosis was induced by bile duct ligation (BDL). Histopathological changes were evaluated by hematoxylin and eosin staining, and Masson's trichrome method. ERK₁ mRNA in rat liver tissue was determined by reverse transcription-polymerase chain reaction, while the distribution of ERK₁ was assessed by immunohistochemistry. ERK₁ protein was detected by Western blotting analysis. The number of activated HSCs was quantified after alpha smooth muscle actin (α -SMA) staining.

RESULTS: With the development of hepatic fibrosis, the positive staining cells of α -SMA increased obviously, and mainly resided in the portal ducts. Fiber septa and perisinuses were accompanied with proliferating bile ducts. The positive staining areas of the rat livers in model groups 1-4 wk after ligation of common bile duct ($12.88\% \pm 2.63\%$, $22.65\% \pm 2.16\%$, $27.45\% \pm 1.86\%$, $35.25\% \pm 2.34\%$, respectively) were significantly larger than those in the control group ($5.88\% \pm 1.46\%$, $P < 0.01$). With the development of hepatic fibrosis, the positive cells of ERK₁ increased a lot, and were mainly distributed in portal ducts, fiber septa around the bile ducts, vascular endothelial cells and perisinusoidal cells. Western blotting analysis displayed that the expression of ERK₁ and ERK₂ protein was up-regulated during the model course, and its level was the highest 4 wk after operation, being 3.9-fold and 7.2-fold higher in fibrotic rat liver than in controls. ERK₁ mRNA was expressed in normal rat livers as well, which was up-regulated two days after BDL and reached the highest 4 wk after BDL. The expression of ERK₁ was positively correlated with α -SMA

expression ($r = 0.958$, $P < 0.05$).

CONCLUSION: The expression of ERK₁ protein and mRNA is greatly increased in fibrotic rat liver tissues, which may play a key role in HSC proliferation and hepatic fibrogenesis.

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Key words: Extracellular signal-regulated kinase; Hepatic fibrosis; Hepatic stellate cells; Proliferation

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INTRODUCTION

Mitogen-activated protein kinase (MAPK) pathway is an important intracellular signal transduction system^[1], and extracellular signal-regulated kinase 1 (ERK₁) is the critical and classical pathway of MAPK and plays an important role in several physiological phenomena, including cell local adhesion, migration, proliferation, differentiation, apoptosis and cell cycle^[2]. Hepatic stellate cells (HSCs) play a pivotal role in hepatic fibrogenesis^[3-6]. In normal liver, HSCs are perisinusoidal mesenchymal elements characterized by intracytoplasmic lipid droplets rich in retinyl esters. On the contrary, in chronic liver injury, HSCs undergo a response known as "activation", which is the transition of quiescent cells into proliferative, fibrogenic and contractile myofibroblasts^[7-9].

Up till now, many studies have focused on the role of MAPK in various cultured cells^[10,11], little is known about its regulation *in vivo*^[12]. No report on dynamic expression of ERK₁ mRNA in fibrotic liver tissue is available. To probe into the molecule mechanism of fibrogenesis by ERK₁, the changes in distribution and contents of ERK₁ protein and mRNA in rat hepatic fibrogenesis were observed by immunohistochemistry, Western blotting and reverse transcription-polymerase chain reaction (RT-PCR). The dynamic expression of α -smooth muscle (α -SMA) as a marker of activated HSCs was also examined by immunohistochemistry in this study.

MATERIALS AND METHODS

Reagents

The monoclonal antibodies against ERK₁ and α -SMA were products of Santa Cruz Biotech Inc. Streptavidin-peroxidase immunohistochemical kit was purchased from Zhongshan Biological Technology Co (Beijing). Trizol reagent was obtained from Life Technologies Inc (USA). One tube RT-PCR kit was from Promega Co (USA). Primers for rat ERK₁ and β -actin were designed by ourselves in accordance with gene sequence in Genbank, synthesized and purified by Bao Biological Engineering Co (Dalian). All other reagents were analytically pure.

Animal model and experimental protocol

A total of 80 adult male Sprague-Dawley rats weighing 350–400 g were purchased from the Experimental Animal Center of Hebei Medical University (Clearing Grade, Certificate No. 04057). All rats were housed in plastic cages with free access to food and water. The rats were randomly divided into 8 groups (10 rats in each group). The rats were subjected to laparotomy with complete ligation of the common bile duct and received ketamine hydrochloride at a dose of 100 mg/kg by intraperitoneal injection^[13]. Under deep anaesthesia, the peritoneal cavity was opened and the common bile duct was double-ligated with 3-0 silk and cut between the ligatures. Control animals underwent a sham operation that consisted of exposure but not ligation of the common bile duct. At various intervals post-operatively, the animals were anaesthetised and the livers were harvested. Liver tissue specimens were routinely fixed in 10% phosphate-buffered formaldehyde and embedded in paraffin. Some liver tissue specimens were used for light microscopy and immunohistochemistry by using anti- α -SMA and ERK₁, while others were snap-frozen in liquid nitrogen and stored at -80°C for RNA analysis. In addition, control livers were harvested 4 wk after sham operation.

Histopathology

Liver specimens were routinely fixed overnight in 10% phosphate-buffered formaldehyde, embedded in paraffin for light microscopic examination. Tissue sections (5- μ m thick) were stained with haematoxylin and eosin (H&E) for morphological evaluation and Masson's trichrome for assessment of fibrosis.

Immunohistochemical detection of α -SMA and ERK₁

All immunohistochemical studies using the streptavidin-peroxidase technique were performed on 5- μ m thick paraformaldehyde-fixed and paraffin-embedded liver tissue sections mounted on APES-coated slides. Slides were deparaffined in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was quenched with a 3% hydrogen peroxide solution in methanol at room temperature for 30 min, followed by rinsing in pH 6.0 phosphate-buffered saline (PBS). After antigen retrieval in a water bath set in a 10 mmol/L citrate buffer (pH 6.0) at 94°C for 8 and 10 min, respectively, the slides were immediately cooled for 20 min at room temperature. Non-specific binding sites were blocked by incubation with wash buffer containing 10% normal goat serum at 37°C for 30 min.

The sections were then incubated overnight at 4°C with a mouse monoclonal antibody directed against α -SMA or a rabbit monoclonal antibody directed against ERK₁ at a dilution of 1:100. The secondary antibody bindings were localized using a biotin-conjugated rabbit anti-mouse IgG for α -SMA and goat anti-rabbit IgG for ERK₁ (1:100 dilution), followed by incubation with streptavidin-peroxidase complex (1:200 dilution). Peroxidase conjugates were subsequently visualised by utilizing diaminobenzidine (DAB) solution in hydrogen peroxide as a chromogen yielding a brown reaction product. The sections were then counterstained with Mayer's hematoxylin and mounted on a cover slip. All incubations were performed in a moist chamber. Furthermore, between each incubation step, the slides were washed 3 times with PBS for 5 min. To ensure the specificity of antibody, negative control samples were processed in parallel under the same conditions but with omission of the first antibody, which was replaced by an equal volume of PBS. The α -SMA and ERK₁ positive parenchymas were measured by a video-image analysis system and expressed as a percentage of area occupied by the signal.

Preparation of cell lysates and Western blotting analysis

Liver tissues from control and bile duct ligation (BDL) animals were quickly removed and washed twice with ice-cold PBS, and then homogenized in modified radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris-HCl, pH 7.5; 150 mmol/L NaCl; 1% Nonident P-40; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate; 1 mmol/L EDTA; 1 mmol/L PMSF; 2 μ g/mL 1 \times leupeptin) for 30 min at 4°C, followed by rotating the tubes at 12000 \times g at 4°C for 10 min. After centrifugation, cleared tissue lysates were collected and stored at -80°C and protein concentration of each sample was determined by Coomassie brilliant blue protein assay. Each sample was adjusted up to a desired protein content of 150 μ g, an equal volume of 5 \times SDS loading buffer was added and the sample was incubated at 100°C for 3 min. Lysate containing 150 μ g of protein was separated by electrophoresis on 10% acrylamide sodium dodecyl sulfate (SDS) gels and transferred onto nitrocellulose membranes. After blocked in a buffer (pH 7.2) containing 1% bovine serum albumin and 5% skim milk powder, the membranes were incubated with rabbit anti-ERK₁ antibodies diluted at 1:600 overnight at 4°C, then incubated for 2 h at 37°C with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody diluted 1:5000 in a blocking buffer. After washed 3 times with PBS containing 0.1% Tween 20, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system on radiograph film. The intensity of the bands was determined by scanning video densitometry. Experiments were performed at least 3 times with similar results.

RNA extraction and RT-PCR assay

Expression of ERK₁ mRNA was evaluated by RT-PCR. Total RNA from liver specimens (100 mg) was isolated using a monophasic solution of phenol and guanidine thiocyanate (Trizol), precipitated in ethanol and resuspended in sterile RNAase-free water for storage at -80°C until use, as recommended by the suppliers. Total

RNA was quantified spectrometrically at 260 nm, and the quality of isolated RNA was analysed on agarose gels under standard conditions. One-step RT-PCR was performed according to the manufacturer's instructions. Two-microgram RNA was added to each reaction and RT-PCR was routinely performed utilizing 5 units of AMV reverse transcriptase, 5 units of *T*₇ DNA polymerase, 10 pmol of each oligonucleotide primer, 10 pmol of dNTP mix and 25 mmol/L MgSO₄ in a final reaction volume of 50 μ L. Primer sequences were as follows: ERK₁: forward 5'-GCT GAC CCT GAG CAC GAC CA-3' and reverse 5'-CTG GTT CAT CTG TCG GAT CA-3', fragment length 451 bp; β -actin: forward 5'-AGC TGA GAG GGA AAT CGT GCG-3' and reverse 5'-GTG CCA CCA GAC AGC ACT GTG-3', fragment length 300 bp. RT-PCR was performed in the following steps: reverse transcription at 41°C for 45 min, pre-denaturation at 94°C for 2 min. Then amplification was performed in a thermal controller for 35 cycles (denaturation at 94°C for 40 s, annealing at 52°C for 1 min, extension at 72°C for 1.5 min, and a final extension at 72°C for 10 min). Ten μ L of the PCR products was analyzed by 1.5% agarose gel electrophoresis with TAE buffer at 80 V for 40 min, visualized with ethidium bromide staining and photographed under UV illumination. The band intensities were quantified by densitometry. ERK₁/ β -actin quotient was the indication of ERK₁. Experiments were performed at least 3 times with similar results.

Statistical analysis

The data were expressed as mean \pm SD. Group means were compared by using analysis of variance followed by the Student-Newman-Keuls test if the former was significant. The correlation between the expressions of ERK₁ and α -SMA was analyzed for statistical significance by the simple linear regression analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Histology of progressive fibrotic liver injury

In the present study, spotted (or scattered) perivenular degeneration of hepatocytes, increased inflammatory infiltrate in the necrotic areas and bile ductular proliferation in the portal triads were observed after 1 wk of BDL. After 2 wk of BDL, all rats showed expanded portal tracts with fibrous tissue, portal-to-portal fibrous bridging, nodular transformation and widespread proliferating bile ductules which extended into the parenchyma in places, without clear-cut cirrhosis. After 3-4 wk of BDL, the animals developed severe fibrosis associated with proliferating bile ducts which formed a continuous meshwork of connective tissue with complete distortion of lobular architecture, whereas there was no histological abnormality or evidence of stainable collagen in any of the sham-operated control livers.

Identification of proliferating and activated HSCs

Immunostaining for α -SMA was used to detect and quantify the number of activated HSCs in this study. The α -SMA positive cells in the sham-operated control

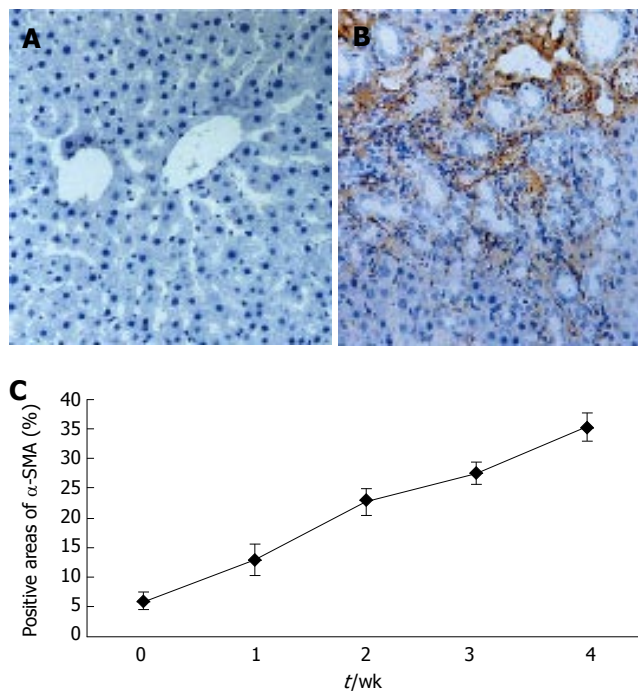


Figure 1 α -SMA protein expression in immunohistochemically-stained liver tissue (SP \times 200). **A:** Few α -SMA expressions in sham operation group; **B:** Positive α -SMA cells residing in the cells of portal ducts, fiber septa, perisinuses and around the proliferated bile ducts 2 wk after BDL; **C:** Positive areas of α -SMA expression in model groups at wk 1 to 4 after common bile duct ligation.

livers were observed in vascular smooth muscle cells and sinusoids with a weak staining. With the development of hepatic fibrosis, the number of positive α -SMA cells was greatly elevated and mainly resided in the cells of portal ducts, fiber septa, and perisinuses accompanied with proliferating bile ducts. The positive areas of the rat livers were larger in model groups at 1 to 4 wk (12.88% \pm 2.63%, 22.65% \pm 2.16%, 27.45% \pm 1.86%, 35.25% \pm 2.34%) than in control group (5.88% \pm 1.46%) ($P < 0.01$, Figure 1A-1C).

Distribution of ERK₁ protein in bile duct-ligated rat liver tissues

To explore the distribution of ERK₁, the normal and fibrotic rat liver tissue sections were immunostained using specific monoclonal anti-ERK₁ antibody. ERK₁ was found in vascular endothelial and perisinusoidal cells of normal rat liver tissue sections. With the development of hepatic fibrosis, the positive ERK₁ cells increased a lot and were mainly distributed in portal ducts, fiber septa, around the bile ducts, as well as in vascular endothelial and perisinusoidal cells and hepatocytes. ERK₁ protein was expressed not only in cytoplasm mentioned above, but also in nuclear membrane, indicating its activation. The positive areas of rat livers in model groups at 1 to 4 wk (9.58% \pm 1.01%, 17.43% \pm 1.78%, 23.88% \pm 2.97%, 28.63% \pm 2.72%) were larger than those in control group (5.03% \pm 1.10%) ($P < 0.01$, Figure 2A-2C).

Expression of ERK₁ protein in common bile duct-ligated rat livers

Western blot analysis showed that ERK₁ was expressed in normal rats with a prolonged model-making period.

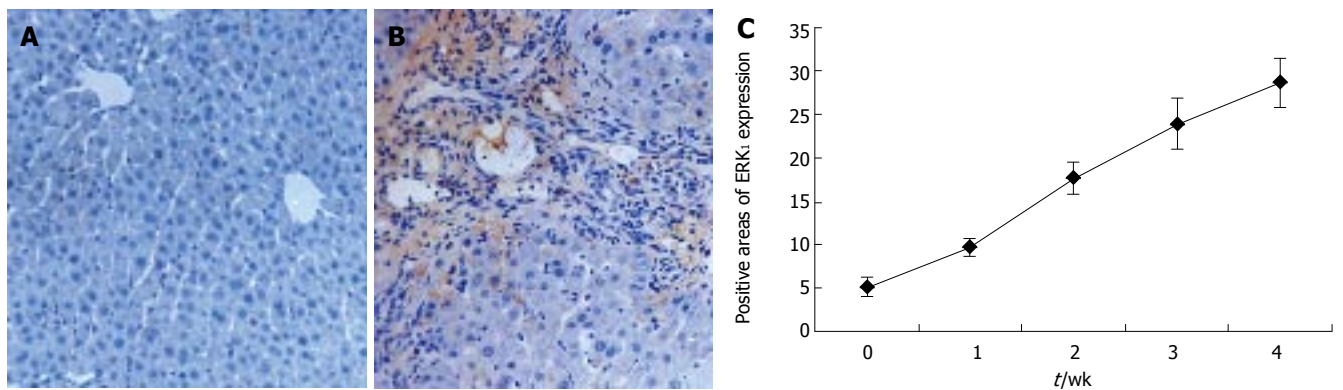


Figure 2 ERK₁ distribution in immunohistochemically-stained liver tissue (SP × 200). **A**: ERK₁ distribution in sham operation group; **B**: ERK₁ distribution 2 wk after BDL; **C**: The time course of ERK₁ distribution in hepatic fibrogenesis.

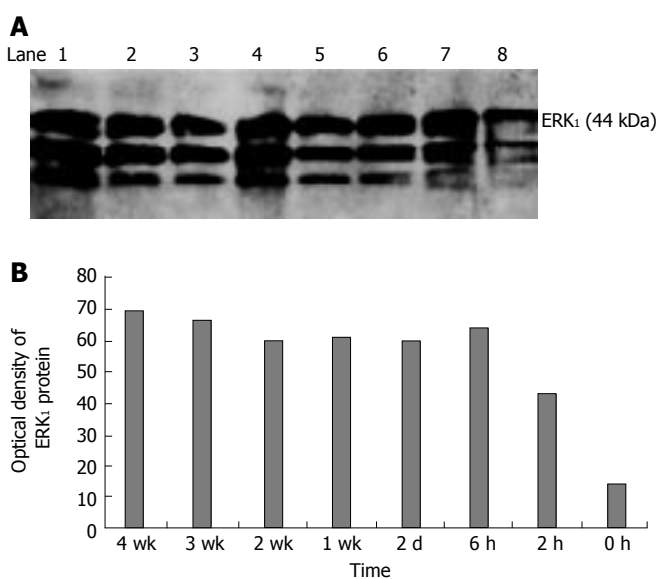


Figure 3 Time course of ERK₁ protein expression (**A**) and quantitative results (**B**) in fibrotic and normal rat liver tissues by Western blotting analysis. 1: 4 wk after BDL; 2: 3 wk after BDL; 3: 2 wk after BDL; 4: 1 wk after BDL; 5: 2 d after BDL; 6: 6 h after BDL; 7: 2 h after BDL; 8: Sham operation group.

The expression of ERK₁ increased 3.07-, 4.51-, 4.36-, 4.36-, 4.36- and 4.71-fold at 2, 6 h; 2 d; 1, 2 and 3 wk; respectively, and reached the peak at 4 wk, which was 4.93 times of the expression in sham-operated group ($P < 0.01$, Figure 3A and 3B).

Increased ERK₁ mRNA expression in bile duct-ligated rat livers

Although ERK₁ protein is produced by liver tissue *in vivo*, it is not clear whether the ERK₁ mRNA level under fibrogenic response is increased *in vivo*. Therefore, we investigated the production of ERK₁ mRNA in liver. Total liver RNA was analyzed by RT-PCR. The results revealed faint transcript of ERK₁ mRNA in sham-operated rat livers. However, specific banding to ERK₁ mRNA was detected in fibrotic liver sections. Moreover, ERK₁ mRNA expression was initially up-regulated at 6 h and reached its peak level 4 wk after BDL, which increased 2.4-fold. Levels of the housekeeping gene, β -actin, did not show

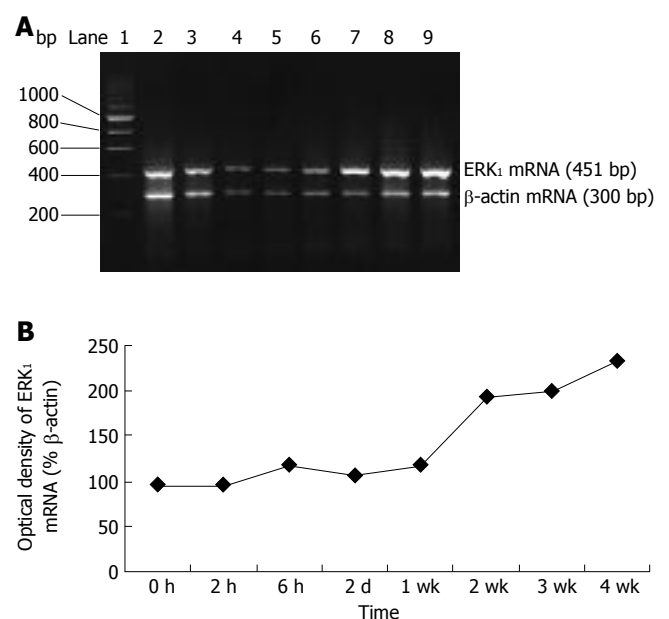


Figure 4 Electrophoresis of RT-PCR for ERK₁ mRNA expression in hepatic fibrogenesis at different time points (**A**) and percentage of optical density of ERK₁ mRNA electrophoretic strip (**B**). 1: marker; 2: sham operation group; 3: 2 h after BDL; 4: 6 h after BDL; 5: 2 d after BDL; 6: 1 wk after BDL; 7: 2 wk after BDL; 8: 3 wk after BDL; 9: 4 wk after BDL.

any significant differences between control and BDL rat liver tissue (Figure 4A and 4B).

Analysis of relation between ERK₁ and α -SMA

Immunohistochemistry experiments were performed to analyze whether the above described ERK₁ protein distribution was correlated with α -SMA in sham operated livers compared with BDL rats. The results indicated that ERK₁ was positively correlated with α -SMA ($r = 0.958$, $P < 0.05$).

DISCUSSION

HSCs, the principal cellular source of extracellular matrix during chronic liver injury, undergo a transition into α -SMA-expressing myofibroblastlike cells in

response to various liver injuries such as ECM, cytokine, inflammation mediator, ethanal, oxygen radical and lactic acid. Furthermore, HSC activation is associated with stellate cell proliferation, increased contractility and enhanced matrix production. HSCs express a number of fibrogenic and proliferative cytokines and their cognate receptors. Therefore, HSCs play a crucial role in cellular and molecular events that lead to hepatic fibrosis^[4]. Cassiman *et al*^[13] and Ramm *et al*^[14] have demonstrated that positive α -SMA cells mainly reside in the portal ducts and fiber septa, accompanied with proliferating tubercle, corresponding to the distribution of collagen. This study displayed that with the development of hepatic fibrosis, the positive α -SMA cells were greatly elevated and mainly situated in cells of the portal ducts, fiber septa, perisinuses accompanied with proliferating bile ducts. The positive areas of α -SMA in rat livers of model groups at 1 to 4 wk increased in turn and were larger than those in control group ($P < 0.01$). The result is in accordance with the above reports and indicates that the level of liver fibrosis can respond to α -SMA.

The activation and proliferation of HSCs may be regulated by various factors and signal transduction pathways^[7,15]. MAPK is a group of cytoplasmic serine/threonine kinases distributed extensively in cytoplasm, as a point of convergence of extracellular signal causing nuclear reaction^[1]. It becomes activated after threonine and tyrosine are phosphorylated altogether. In the MAPK family, ERK has been identified. ERK subset includes 2 subtypes: ERK₁ (P44^{MAPK}) and ERK₂ (P42^{MAPK}). The activation pathway is RAS-RAF-MEK-ERK. The combination of extracellular stimulation signal and receptors in cell membrane provokes dimerization of receptors and then phosphorylates self residue-Tyr. Some adapter proteins such as (growth factor receptor bound₂ (Grb₂) are recruited. Grb₂ is composed of one SH₂ region and two SH₃ regions. The SH₃ region of Grb₂ can combine with son of sevenless, which provokes the change of Ras's GDP and GTP. RAS activates RAF kinases further and phosphorylated RAF activates mitogen-activated protein kinase kinase (MEK), which can phosphorylate Thr and Tyr residues of ERK_{1/2}. At last, activated ERK enters nuclear membrane to regulate gene transcription and provokes cell biologic effects.

The role of ERK in promoting HSC proliferation *in vitro* has been increasingly acquainted in recent years. As mentioned earlier, it has been suggested that many cytokines, such as platelet derived growth factor (PDGF), endothelin (ET), epidermal growth factor (EGF), insulin, insulin-like growth factor-1 (IGF-1) and tumor necrosis factor (TNF), as well as endotoxin, ethanol, acetaldehyde, ROS and ECM, can all activate HSCs and promote the production of ERK^[10,16-19]. PD98059, an inhibitor of MEK kinase which is a upstream signal molecule of ERK, can restrain the transformation of HSCs into myofibroblasts, HSC proliferation and collagen synthesis^[14, 20-22]. The study on HSC-deleting MAPK gene *in vitro* discovered that the collagen gene expression of HSCs decreases to one third of the primary^[23]. On the contrary, little is known about the role and regulation mechanism of ERK *in vivo*. In addition, the mechanism of ERK mRNA is unknown. Nguyen *et al*^[24] have demonstrated that the expression of ERK₁ and

ERK₂ in patients with alcoholic liver disease increases 3.9- and 3.2-fold respectively, compared with that in normal people. By using immunohistochemistry, Alvaro *et al*^[25] have confirmed that the activated ERK_{1/2} in rat liver enters nuclear membrane from cytoplasm, and the expression coincides with liver pathology, suggesting that ERK₁ protein and ERK₁ mRNA are scarcely expressed in normal rat livers. In addition, ERK₁ protein and ERK₁ mRNA expressions are elevated with the progression of hepatic fibrosis. The position distribution demonstrates that ERK₁ protein is scarcely expressed in vascular endothelial and perisinusoidal cells in normal rat liver. With the development of hepatic fibrosis, the positive ERK₁ cells are mainly resided along with HSCs, in portal ducts, fibrotic septa, and epithelium of bile ducts. ERK₁ protein is expressed not only in plasma of cells, but also in nuclearmembrane, indicating its activation. Based on these results, we presume that hepatocytes, vascular endothelial cells, epithelium of bile ducts and HSCs are all cellular sources of ERK₁. However, the major cellular source is HSCs during hepatic fibrosis. ERK₁ in the MAPK family probably participates in the development of hepatic fibrogenesis.

In conclusion, ERK₁ protein distribution is positively correlated with α -SMA. ERK₁ is associated with HSC proliferation. Activation of ERK₁ in rat liver tissue can activate the downstream signal molecule which modulates gene expression of HSCs and gives rise to hepatic fibrosis.

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RAPID COMMUNICATION

Is delayed gastric emptying so terrible after pylorus-preserving pancreaticoduodenectomy? Prevention and management

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Abstract

AIM: To explore some operative techniques to prevent the occurrence of delayed gastric emptying (DGE) after pylorus-preserving pancreaticoduodenectomy (PPPD).

METHODS: One hundred and eighty-six patients in a single medical center who accepted PPPD were retrospectively studied. The incidence of DGE was investigated and the influence of some operative techniques on the prevention of DGE was analyzed.

RESULTS: During the operative process of PPPD, the methods of detached drainage of pancreatic fluid and bile and gastric fistulization were used. Postoperatively, six patients suffered DGE among the 186 cases; the incidence was 3.23% (6/186). One of them was complicated with intraabdominal infection at the same time, and two with pancreatic leakage.

CONCLUSION: Appropriate maneuvers during operation are essential to avoid postoperative DGE in PPPD. The occurrence of DGE is avoidable. It should not be used as an argument to advocate hemigastrectomy in PPPD.

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Key words: Delayed gastric emptying; Pylorus-preserving pancreaticoduodenectomy; Gastric fistulization

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INTRODUCTION

Pylorus-preserving pancreaticoduodenectomy (PPPD) was first reported by Watson in 1944^[1] and reintroduced by Traverso and Longmire in 1978^[2]. For the sake of functional preservation of the stomach and improvement of life quality, it has been accepted and adopted by more and more surgeons. With the wide use of PPPD, delayed gastric emptying (DGE) becomes the most common complication in the early postoperative course. Although there is no generally accepted definition of DGE, it may be described as the need for gastric suction during postoperative 7-10 d. Despite the self-limited characteristics of DGE, it prolongs the hospitalization days and increases the sufferings of patients. Meanwhile, it also affects the confidence of surgeons in further use of PPPD. In order to reduce the risk of DGE, we have adopted some effective approaches during operative process, as well as some postoperative managements and gained satisfactory results.

MATERIALS AND METHODS

Patients

From March 1992 to December 2003, 186 consecutive patients from the Department of Hepatobiliary Pancreatic Surgery of our hospital underwent PPPD performed by the same surgical group. Of these patients, 110 were male (59.1%) and 76 were female (40.9%), with a median age of 55.3 years (range, 32 to 83). The most frequent indication was peri-ampullary carcinomas (184 patients) (Table 1).

Operative techniques

Blood supply of duodenal residue should be well preserved: When the hepatogastric ligament and right part of gastroduodenal ligament were resected, the vessel arch around the stomach should be preserved. The right gastric artery and gastroduodenal artery were divided and ligated at their origins to keep the distal arcade. The duodenum was transected 2 to 3 cm below the pylorus. The stomach was packed with wet gauze to protect it from loss of fluid. If the blood supply of residual duodenum was poor, PPPDs were substituted by standard pancreaticoduodenectomy (PD).

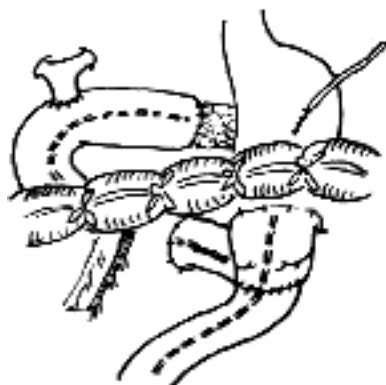


Figure 1 Intubation in pancreatic duct and gastric fistulization.

The detached drainage of pancreatic fluid and bile was performed: After the neck of pancreas was transected, a thin drainage tube (about 3 mm in diameter and 40 cm in length) was inserted into the distal pancreatic duct (Figure 1). The tube could drain the pancreatic fluid out of peritoneal cavity before pancreaticojejunostomy. When the posterior wall of pancreaticojejunostomy was completed, the thin tube was put into the jejunum to drain the pancreatic fluid away from the pancreaticojejunostomy and cholangiojejunostomy. Several small steel balls were put into the distal end of the tube, and their gravity may keep the tube from bending in the jejunum. We named the drain method detached drainage of pancreatic fluid and bile. Regarding the alimentary reconstructive procedures, Child's method was adopted.

Gastric fistulization tube was put into the intestine through the duodejejunal anastomosis: After the posterior wall of duodejejunectomy was sutured, gastric fistulization was made routinely. A small hole was made in the anterior wall of the stomach, and a thin tube (about 40 cm in length and 4 mm in diameter) was inserted through the hole and pylorus to the proximal jejunum. The other end of the tube passed through the abdominal wall and was fixed to the skin. Two abdominal drainage tubes were essential for the operation, one was placed behind the pancreaticojejunostomy and choledochojejunostomy, and the other was placed in front of the pancreaticojejunostomy.

Peri-operative managements

In order to help the patients recover well, relatively stable internal environment and good nutritional condition of the patients should be maintained. In the preoperative and early postoperative stages, correction of imbalance of water and electrolytes and appropriate nutritional support should be undertaken. In most cases, total parenteral nutrition was needed.

The drainage tubes must be carefully watched to keep fluent in postoperative stage, because sometimes blood clots and necrotic tissues may obstruct them. If postoperative abdominal infection was suspected or proved, effective therapies such as drainage of the infectious focus and prescription of antibiotics should be taken promptly. Gastric suction may be stopped within postoperative 2 to 3 d; meanwhile, gastrointestinal motility

Table 1 The types of disease

Type of disease	Cases (n)	%
Ampulla carcinoma	78	41.9
Distal carcinoma of common bile duct	61	32.8
Duodenal carcinoma	4	2.1
Pancreatic head carcinoma	39	21.0
Cyst adenocarcinoma	2	1.1
Chronic pancreatitis	2	1.1

Table 2 Complications after PPPD in this series of cases

Complication	Cases (n)
Delayed gastric emptying	6
Pancreatic leakage	4
Intraabdominal infection	2
Intraabdominal hemorrhage	2
Liver function failure	2
Bile leakage	1

drugs may be taken orally. After the recovery of alimentary function, the patients may have liquid diet, and the amount of the intravenous fluid may be reduced. The use of antacid drugs was routine.

If the DGE was suspected, some examinations were helpful. The first was X-ray examination of the stomach; 76% of meglucamine diatrizoate was injected into the stomach by gastric suction tube. The typical manifestation of DGE was the decrease or diminish of gastric peristalsis; the contrast media retained in the stomach and could not go into the jejunum. A further choice was gastroscopic examination; the obstruction of gastrojejunostomy might be excluded. To improve the DGE, nutritional support was essential. Because of the thin tube in the stomach and jejunum; enteral nutrition was available and convenient. Small doses of erythromycin (0.6-0.9 g/d) might be administered intravenously. Some other drugs might also be used to stimulate the peristalsis of gastrointestinal tract, such as cisapride. For some patients, the change of body position was helpful, such as lying on the right side.

Therapeutic results

The complications of PPPD are listed in Table 2. Six patients suffered DGE among the 186 cases; the incidence was 3.23% (6/186). Among them, one was also complicated with intraabdominal infection simultaneously, and two with pancreatic leakage. The median recovery time was 36 (range: 15-81) d. One patient recovered after the gastroscopic examination. Three patients became normal after the use of small doses of erythromycin and cisapride. One patient did not show remission though all of the above methods were tried. However, at the 81st day postoperation, the uncomfortable symptoms of the patient disappeared spontaneously.

DISCUSSION

As the main complication of PPPD, DGE may occur

in 20%-60% of patients^[3-8]. The pathogenesis of DGE is still unknown. Thor and his coworkers^[9] found that the operation might damage the enteric nervous system, which induced the profound changes in gastric motility and emptying. Some authors^[9,10] stressed that the ischemic injury of antrum and pylorus plays an important role in the occurrence of DGE. Others emphasized the central role of the change of motilin concentration in plasma in DGE occurrence. All these above factors may partially participate in occurrence of DGE. In clinics, DGE is characterized with gastric dysfunction. The gastrointestinal radiography indicates the stomach is dilated; the gastric peristalsis is decreased or diminished. By the examination of gastroscopy, we may see the duodenojejunostomy anastomosis and gastric mucosa is congested and edematous, but the anastomosis is unobstructed, and the top end of gastroscopy may go through the anastomosis smoothly.

In our series, gastric fistulization was a routine maneuver. We assumed that the thin tube played an important role in prevention of DGE. In the early postoperative period, even if the existence of gastric atony, the gastric juice and liquid food may flow into jejunum along the wall of the tube, similar to the situation of flow of the bile into the duodenum in the patient with endoscopic nasobiliary drainage. Therefore, the gastric retention can be decreased. The corresponding symptoms such as nausea and vomiting may be avoided. Because the tube had many orifices in the walls at its forepart, if the gastric retention was very serious, negative pressure suction was convenient through the tube. If the abnormal gastric function lasted for a period of time and led to delayed food ingestion, enteral nutrition was available through the jejunal tube, thus avoiding some complications arising from long-term parenteral nutrition.

Besides the above-mentioned factors that may lead to DGE, postoperative intra-abdominal complications are also an important cause. Riediger^[11] studied 204 patients undergoing PPPD. By multivariate analysis, it was revealed that the postoperative complications were the most important factor associated with the occurrence of DGE. Park^[12] studied 150 consecutive patients who underwent PPPD performed by one surgeon. The incidence of DGE was 41.7% (15 of 36) in the group with complications, yet only 8.8% (10 of 114) in the other group ($P = 0.0001$). Among the postoperative intraabdominal complications, pancreatic leakage was most often seen. In our series, two of the six patients with DGE were complicated with pancreatic leakage. Therefore, it is important to prevent the pancreatic leakage during postoperative period.

Pancreatic leakage is also a primary complication after PPPD. It is far more disastrous than DGE. It is not only related to delayed gastric emptying, but can lead to some fatal consequences such as intra-abdominal bleeding and infection. How to prevent its occurrence is always a compelling problem for surgeons. In our experience, detached drainage of pancreatic fluid and bile is an effective method^[13] for the prevention of pancreatic leakage. The tube in pancreatic duct can drain pancreatic fluid into the jejunum away from the pancreaticojejunostomy. No pancreatic fluid detains in the intestinal lumen near the

pancreaticojejunostomy, the enteral pressure is decreased and pancreaticojejunostomy is free of the soaking and corrosion resulting from pancreatic fluid. Along the outer wall of the tube, the bile is also drained to the distal jejunum away from cholangiojejunostomy where it mixes with pancreatic fluid and activates the digestive proenzyme in pancreatic fluid. Due to adoption of these methods, the incidence of pancreatic leakage was rather low in our series. We believe that it is helpful for the prevention of the DGE. Compared with the traditional method, in which the pancreatic fluid is drained outside body, our technique may decrease the loss of body fluid, thus helpful for maintaining stable internal environment and balancing the water and electrolytes of the patients.

As a postoperative complication characterized by gastrointestinal dysfunction, DGE is not specific for PPPD. It may occur after other abdominal operations, including the standard pancreaticoduodenectomy. Tran studied^[14] a nonselected series of 170 consecutive patients and found DGE occurred equally in the 2 groups. Horstmann^[15] also proposed that pylorus preservation did not increase the frequency of DGE. DGE almost exclusively occurs as a consequence of other postoperative complications. As we know, the best way to avoid postoperative complications is appropriate maneuvers during operation. In our series, besides elaborate surgical techniques, we used the thin tube twice, and the small tubes really play big roles in preventing DGE. Since the occurrence of DGE is avoidable in most cases, DGE should not be used as an argument to advocate hemigastrectomy in PPPD.

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RAPID COMMUNICATION

Preventive effect of tetramethylpyrazine on intestinal mucosal injury in rats with acute necrotizing pancreatitis

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Abstract

AIM: To evaluate the role of microcirculatory disorder (MCD) and the therapeutic effectiveness of tetramethylpyrazine (TMP) on intestinal mucosa injury in rats with acute necrotizing pancreatitis (ANP).

METHODS: A total of 192 Sprague-Dawley rats were randomly divided into three groups: normal control group (C group), ANP group not treated with TMP (P group), ANP group treated with TMP (T group). An ANP model was induced by injection of 50 g/L sodium taurocholate under the pancreatic membrane (4 mL/kg). C group received isovolumetric injection of 9 g/L physiological saline solution using the same method. T group received injection of TMP (10 mL/kg) *via* portal vein. Radioactive bioluminescence technique was used to measure the blood flow at 0.5, 2, 6 and 12 h after the induction of ANP. Samples of pancreas, distal ileum were collected to observe pathological changes using a validated histology score. Intestinal tissues were also used for examination of myeloperoxidase (MPO) expressed intracellularly in azurophilic granules of neutrophils.

RESULTS: The blood flow was significantly lower in P group than in C group ($P < 0.01$). The pathological changes were aggravated significantly in P group. The longer the time, the severer the pathological changes. The intestinal MPO activities were significantly higher in P group than in C group ($P < 0.01$). The blood flow of intestine was significantly higher in T group than in P group after 2 h ($P < 0.01$). The pathological changes were alleviated significantly in T group. MPO activities were significantly lower in T group than in P group ($P < 0.01$ or $P < 0.05$). There was a negative correlation between intestinal blood flow and MPO activity ($r = -0.981$, $P < 0.01$) as well as between intestinal blood flow and pathologic scores ($r = -0.922$, $P < 0.05$).

CONCLUSION: MCD is an important factor for intestinal injury in ANP. TMP can ameliorate the condition of MCD and the damage to pancreas and intestine.

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Key words: Acute necrotizing pancreatitis; Microcirculation; Tetramethylpyrazine; Intestinal mucosal injury

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INTRODUCTION

Acute pancreatitis (AP) is often complicated by intestinal injury. However, its pathogenesis remains unclear. As we know, AP involves a complex array of mediators initiating and amplifying the systemic inflammatory response and therefore leads to failure of the distant organ systems, such as the lungs, intestine and kidneys^[1-3]. Failure of intestinal barrier function often occurs in acute necrotic pancreatitis (ANP), resulting in the increased intestinal permeability and subsequent translocation of bacteria or/and endotoxin from gut^[4]. Recent studies indicate that during the pathogenesis of ANP, changes in microcirculation play an important role in the worsening of pancreatitis^[5]. A number of agents have been tried for preventing or ameliorating this destructive effect on intestine in experiment conditions. Pharmacologic studies have demonstrated that tetramethylpyrazine (TMP), an intravenous drug made from traditional Chinese herbs, is able to inhibit release of intracellular calcium and to scavenge oxygen free radicals^[6,7]. The significant efficacy of TMP in cerebral ischemia and reperfusion injury has been confirmed. This study was to evaluate the role of microcirculatory disorder (MCD) in ANP of rats and the therapeutic effectiveness of TMP on intestinal injury in ANP.

MATERIALS AND METHODS

Animals

Adult Sprague-Dawley rats of both sexes weighing

250-300 g were provided by the Laboratory Animal Center of Jiangsu University. The animals were fed with standard rat chow and water *ad libitum*. The rats were allowed to acclimatize to our laboratory conditions for 1 wk and then subjected to mesh stainless-steel cages at a constant temperature ($21 \pm 1^\circ\text{C}$) in a 12 h day/night cycle. Prior to experiment, the rats were fasted overnight with free access to water.

Experimental design

The animals were randomly divided into control group (C group), ANP group not treated with TMP (P group), ANP group treated with TMP (T group) with 64 rats in each group. Each group was further divided into 0.5, 2, 6 and 12 h subgroups, respectively. The rats were anaesthetized with sodium pentobarbital (50 mg/kg, ip) and operated under aseptic conditions. The rats were infused with sodium taurocholate (50 g/L, 4 mL/kg, Ward Blen Kinsop CO) through the pancreatic membrane to induce ANP model as previously described^[8]. After 5-10 min, pancreatic edema and dotted bleeding occurred. The rats in T group were infused with sodium taurocholate. Then 6 g/L TMP (Wuxi No. 7 Pharmaceutical Factory, China; batch number: 0008241) was infused *via* femoral vein (10 mL/kg). The rats in C group received isovolumetric injection of 9 g/L physiological saline solution. The abdominal wounds were closed and all the rats were returned to their cages. Thirty-four animals in each group were sacrificed at 0.5, 2, 6 and 12 h after infusion for further examination. Part of distal ileum was removed immediately and fixed in paraformaldehyde solution for 12-24 h and paraffin-embedded for routine histopathologic analysis and measurement of myeloperoxidase (MPO) activity. The histopathologists were blinded to routine histopathologic analysis. The other rats in each group were used for intestinal blood flow determination by intravenous injection of radioactive microsphere technique (RMT).

Blood flow measurements

At 0.5, 2, 6 and 12 h after infusion, intestinal blood perfusion values were determined using RMT as previously described^[9] and modified by Chen and Dai^[10]. $^{99}\text{Tc}^{\text{m}}$ -labeled microspheres (^{99}Mo - $^{99}\text{Tc}^{\text{m}}$ generator preparation provided by Chinese Institute of Nuclear Power) with a specific activity of 74 MBq/mL were used for measurement of blood flow. The right carotid artery was catheterized with the tip of the catheter placed in the left ventricle for infusion of $^{99}\text{Tc}^{\text{m}}$ -labeled microspheres. One mL $^{99}\text{Tc}^{\text{m}}$ radioactive microspheres (approximately 500 000 microspheres) was injected for 10 s *via* the catheter with its tip in the aortic ventricle of heart. A reference blood sample was obtained from the femoral artery catheter for 60 s at a constant rate of 1 mL/min with a continuous-withdrawal pump. The microsphere animals were killed by intra-arterial injection of 2 mL 100 g/L KCL. The distal ileum was removed, weighed and cut into small pieces and placed in a γ counter (GC-1200 gamma radioimmunoassay counter was from USTC Chuangxin CO.Ltd) to determine the radioactivity. Samples from each animal were counted and expressed as counts per minute (CPM). The blood

flow values were calculated according to the following formula:

$$\text{Qorg [mL/ (min}\cdot\text{g)]} = \frac{\text{Qref (mL/min)} \times \text{Norg (cpm)}}{\text{Nref (cpm)} \times \text{Weighing (g)}}$$

Where Qorg denotes the organ blood flow (mL/min \cdot g), Qref is the withdrawal rate of the reference sample (mL/min), Norg is the number of microspheres in the organ (cpm) and Nref is the number of microspheres in the reference sample (cpm).

Myeloperoxidase activity

Sequestrations of neutrophils within the intestinal mucosa were evaluated by quantifying tissue MPO activity as previously described^[11]. Intestinal tissue was removed and immediately frozen and stored at -80°C until MPO assay with a 753 UV-Vis spectrophotometer (Shanghai Optical Instrument Factory). The results were expressed as nKat/g of tissue.

Pathological examination

The rats were killed by intra-arterial injection of 2 mL 100 g/L KCL. The whole pancreas and parts of distal ileum were obtained and promptly fixed in 40 g/L phosphate-buffered formaldehyde for further studies. Paraffin-embedded tissue sections (5-7 μm thick) were stained with hematoxylin and eosin. Histological evaluation was performed under light microscope by two blinded observers on two separate occasions. Mucosal damage was assessed according to the standard scale of Chiu *et al.*^[12]. Grading was performed and classified as: 0 = normal mucosa; 1 = development of subepithelial space at the tip of the villus; 2 = extension of the space with epithelial lifting; 3 = massive epithelial lifting; 4 = denuded villi; 5 = disintegration of the lamina propria (Figure 1).

Statistical analysis

Results were expressed as mean \pm SD except for data of the grading of intestinal mucosal injury. The results were analyzed using the postHoc test. Differences in grading of intestinal mucosal injury were analyzed with the Mann-Whitney *U* test. The correlation of intestinal blood flow with MPO activity was analyzed using Pearson's correlation. The correlation of pathologic score with the intestinal blood flow was analyzed using Spearman's rank correlation. $P < 0.05$ was considered statistically significant.

RESULTS

Intestinal mucosal blood flow

As shown in Table 1, the blood flow was significantly lower in P group than in C group ($P < 0.01$). It began to decrease at 0.5 h and reached the lowest at 12 h. However, the blood flow in T group was significantly higher than that in P group after 2 h ($P < 0.05$), and significantly different from that in C group ($P < 0.01$).

Myeloperoxidase activity

MPO activity in P group greatly increased compared to that in C group ($P < 0.01$). For T group, Although its MPO

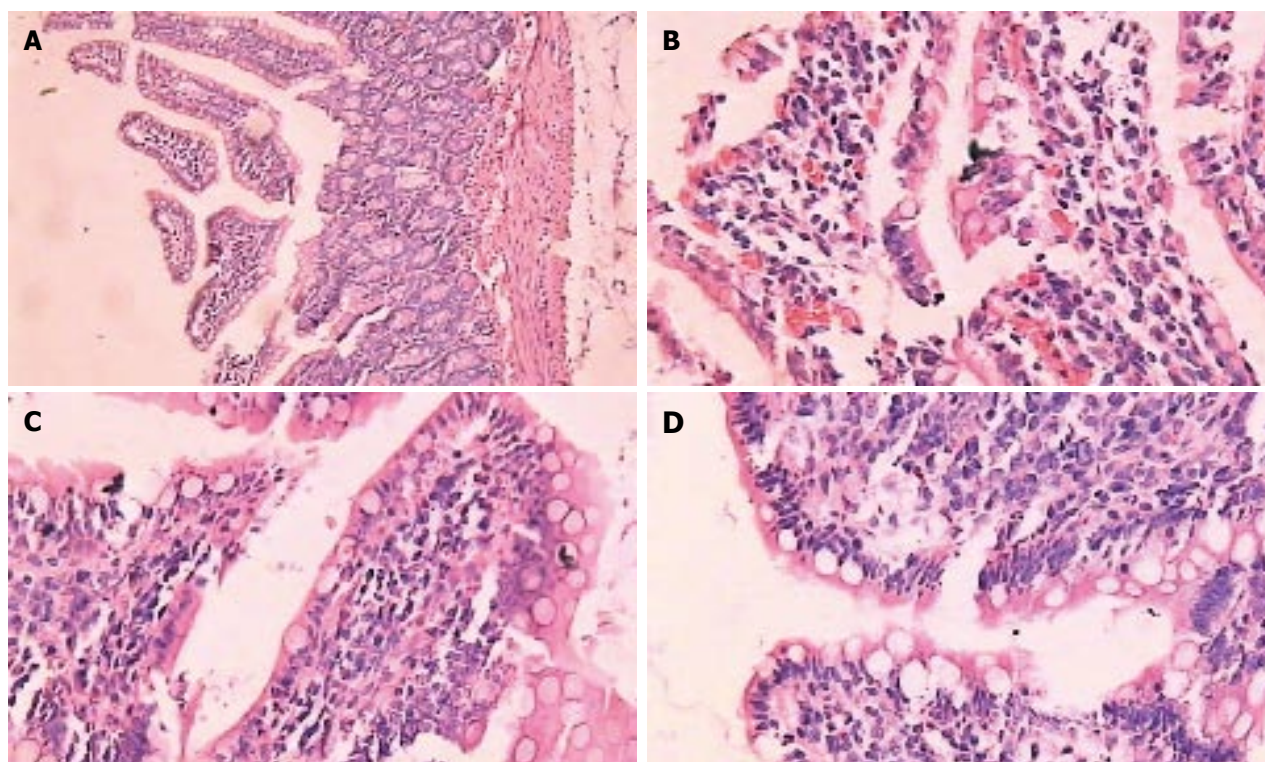


Figure 1 Morphological changes in intestinal mucosa after induction of ANP with/without treatment with TMP (HE \times 100). **A:** Intestinal section with normal mucosa histopathologically graded as 0; **B:** Intestinal section with massive epithelial lifting histopathologically graded as 3; **C:** Intestinal mucosa with denuded villi histopathologically graded as 4; **D:** Little damage to intestinal section treated with TMP.

Table 1 Intestinal blood flow change (mL/min per gram) (mean \pm SD, $n = 8$)

Group	0.5 h	2 h	6 h	12 h
C	1.54 \pm 0.14	1.56 \pm 0.18	1.56 \pm 0.12	1.61 \pm 0.11
P	0.75 \pm 0.07 ^b	0.80 \pm 0.07 ^b	0.63 \pm 0.07 ^b	0.50 \pm 0.06 ^b
T	0.71 \pm 0.14 ^b	1.05 \pm 0.12 ^{b,d}	1.07 \pm 0.12 ^{b,d}	0.92 \pm 0.08 ^{b,d}

^b $P < 0.01$ vs C group; ^d $P < 0.01$ vs P group.

Table 2 Intestinal MPO activity in C, P and T groups (mean \pm SD, nKat/g)

Group	0.5 h	2 h	6 h	12 h
C	58.17 \pm 0.05	57.28 \pm 0.19	58.02 \pm 0.04	68.02 \pm 0.11
P	118.05 \pm 0.25 ^b	119.70 \pm 0.23 ^b	136.52 \pm 0.17 ^b	134.86 \pm 0.21 ^b
T	85.86 \pm 0.35 ^{a,c}	90.21 \pm 0.12 ^{a,c}	107.52 \pm 0.23 ^{b,d}	128.3 \pm 0.12 ^{b,d}

^a $P < 0.05$, ^b $P < 0.01$ vs C group; ^c $P < 0.05$, ^d $P < 0.01$ vs P group.

Table 3 Intestinal mucosal injury in each group ($n = 8$)

Group	0.5 h						2 h						6 h						12 h					
	0	I	II	III	IV	V	0	I	II	III	IV	V	0	I	II	III	IV	V	0	I	II	III	IV	V
C	7	1	0	0	0	0	6	2	0	0	0	0	7	1	0	0	0	0	6	2	0	0	0	0
P	0	2	5	1	0	0 ^b	0	0	2	4	2	0 ^b	0	0	0	1	4	3 ^b	0	0	0	0	3	5 ^b
T	0	3	5	0	0	0 ^{a,d}	3	4	1	0	0	0 ^{a,d}	3	3	2	0	0	0 ^{a,d}	2	3	2	1	0	0 ^{a,d}

^a $P < 0.05$, ^b $P < 0.01$ vs C group; ^c $P < 0.05$, ^d $P < 0.01$ vs P group.

activity was also higher in T group than in control group, it increased less than in P group ($P < 0.01$ or $P < 0.05$, Table 2).

Pathologic examination of intestinal mucosa and pancreas

After induction of ANP model, pancreas showed mild edema and congestion. Two hours after introduction of

the model, typical pathologic changes were found in ANP, such as a large number of inflammatory cells, necrosis of adjacent fat tissues, interstitial edema, parenchyma hemorrhage and necrosis, large amount of ascites. The results had a concordance of 100% of the readings by 2 pathologists. The degree of intestinal pathological injury is shown in Table 3. The grades of P group were

significantly higher than those of control group ($P < 0.01$). The histopathological grades of T group were significantly lower than those of P group ($P < 0.05$) (Figure 1).

Correlation analysis

Correlation analysis showed that there was a negative correlation between intestinal blood flow and MPO activity ($r = -0.981$, $P < 0.01$) as well as between intestinal blood flow and pathologic score ($r = -0.922$, $P < 0.05$).

DISCUSSION

Microcirculatory mechanisms are involved in the development of acute pancreatitis^[13,14]. Changes in microcirculation is an important factor for the development of ANP. It can damage the pancreas and extra pancreatic vital organs^[15,16], leading to a series of changes including vasoconstriction, ischemia, increased vascular permeability, impairment of nutrient tissue perfusion, ischemia/reperfusion injury, leukocyte adherence. RMT provides an efficient method for estimating blood flow to various organs in the body. Our results revealed that, at the early stage of ANP, intestinal mucosal blood flow decreased significantly 0.5 h after injection of 50 g/L sodium taurocholate as compared with control group ($P < 0.01$). Intestinal mucosal injury occurred at the same time which might result from ANP and release of inflammatory mediators. Changes in the nerve endocrine system bring about redistribution of viscera blood flow, producing a sharp decrease in intestinal blood flow. Intestinal mucosa is sensitive to the shortage of blood and oxygen. Due to the further decrease in circulatory blood and over activation of inflammatory mediators, much lower intestinal blood flow further damages the mucosa, suggesting that microcirculation disturbances may contribute to intestinal mucosal damage.

The second prominent feature in this experimental model is the development of granulocytosis and accumulation of neutrophils in intestinal tissue. Because neutrophils are known to be required for the development of intestinal injury in ANP, interventional studies were undertaken to assess whether intravenous administration of TMP would result in decreased neutrophil accumulation in intestinal tissues. The MPO activities in intestinal tissue were assayed to assess the number of recruited neutrophils to the intestinal tissue in C, P and T groups. Our results showed that the MPO activity was markedly elevated after induction of pancreatitis and TMP significantly reduced neutrophil accumulation in intestinal tissue, suggesting that TMP attenuates the increase in MPO activity and improves the histological findings in both pancreas and intestine.

TMP is widely used in traditional Chinese medicine, often in combination with other herbal medicines. It is traditionally used to treat a diversity of ailments, particularly cardiovascular disorders such as atherosclerosis or blood clotting abnormalities. TMP can intervene in hemorheological events in the organs, such as blood flow, erythrocyte deformation, leukocyte adhesion, platelet aggregation and thrombolysis^[17]. TMP protects against vascular endothelial cell injuries induced by hydrogen peroxide^[18] and tissue injuries caused by activated

neutrophils by scavenging oxygen radicals^[19], as well as enhances the action against free radicals and inhibits ca function, which may be one of the mechanisms underlying the protection of cells against intestinal ischemic reperfusion injury^[20].

In conclusion, TMP attenuates MPO activity and improves intestinal microcirculation. Its protective effects against intestinal injury may be attributed to improving microcirculation and further preventing accumulation of neutrophils. MCD plays an important role in the development of intestinal injury. Early use of TMP seems to be effective in ameliorating intestinal injury.

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Aberrant cytological localization of p16 and CDK4 in colorectal epithelia in the normal adenoma carcinoma sequence

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Abstract

AIM: To study the correlation between the patterns of subcellular expression of p16 and CDK4 in colorectal epithelia in the normal-adenoma-carcinoma sequence.

METHODS: Paraffin sections of 43 cases of normal colorectal epithelia and corresponding adenomas as well as carcinomas were analysed immunocytochemically for subcellular expression of p16 and CDK4 proteins.

RESULTS: Most carcinomas showed more cytoplasmic overexpression for p16 and CDK4 than the adenomas from which they arised or the adjacent normal mucosa. Most normal or non-neoplastic epithelia showed more p16 and CDK4 expression in the nucleus than their adjacent adenomas and carcinomas. There was a significant difference between the subcellular expression pattern of p16 and CDK4 in normal-adenoma-carcinoma sequence epithelia ($P < 0.001$). Neither p16 nor CDK4 subcellular patterns correlated with histological grade or Dukes' stage.

CONCLUSION: Interaction of expression of p16 and CDK4 plays an important role in the Rb/p16 pathway. Overexpression of p16 and CDK4 in the cytoplasm, as well as loss expression of p16 in the nucleus might be important in the evolution of colorectal carcinoma from adenoma and, of adenoma from normal epithelia.

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Key words: Colorectal neoplasm; p16; CDK4; Immunocytochemistry

Zhao P, Mao X, Talbot IC. Aberrant cytological localization of p16 and CDK4 in colorectal epithelia in the normal adenoma

INTRODUCTION

p16, as a cyclin-dependent kinase-4 inhibitor is expressed in a limited range of normal tissues and tumors. The Rb/p16 tumor-suppressor pathway is frequently abrogated in many types of human tumors, either through inactivation of Rb or p16 tumor-suppressor proteins, or through alteration or overexpression of cyclin D1 or cyclin-dependent kinase 4/6 (CDK4/6) oncogenes^[1-4]. However, no deletion and only quite a low frequency of mutation of p16 gene have been found in colorectal cancer and adenoma-carcinoma-sequence since this gene was identified in 1994^[1,5]. CpG islands are areas rich in CpG dinucleotides, which are found within the promoters of about 60% of human genes. These CpG islands normally lack DNA methylation, regardless of the expression status of the gene^[6]. Methylation of promoter usually leads to irreversible inhibition of gene transcription^[7]. It has become apparent that *de novo* methylation is an important alternate mechanism underlying coding region mutation which inactivates tumor suppressor genes during neoplasia^[8-12]. Methylation of the 5' CpG island in p16 gene was previously reported not only in colorectal cancer but also in normal colonic tissue by the more sensitive PCR-based assay, in which all p16 genes were inactivated^[9,10]. Furthermore, it has been found that the aberrant cytoplasmic expression of the p16 protein without gene alteration is associated with accelerated tumor proliferation in breast cancer^[13], and p16 protein plays an important role in the regulation of glioma angiogenesis, suggesting a novel function of the p16 gene^[14]. It was also reported that overexpression of p16 was observed in a majority of colorectal cancer^[15-17] and a low p16 expression due to methylation^[18] or not^[15,19,20] may contribute to tumor enlargement, expansion and metastasis and even prognosis of colorectal cancer. Immunohistochemistry with p16 antibody has been used as a diagnostic adjunct in premalignant and malignant lesions of gynecologic pathology^[21], oral cavity^[22,23], head and neck^[24-26] and skin^[27,28] in recent years. Diffuse positivity but not focal one with p16 staining in the cervix can be regarded as a surrogate marker of the presence of

high-risk human papillomavirus (HPV)^[29-31]. Most high-grade cervical intraepithelial neoplasia (CIN) and some cases of low-grade CIN, are usually associated with high-risk HPV. In cervical squamous lesions, the expression of p16 is found almost diffusely positive. And also p16 expression may be helpful to identify small focal high-grade CIN lesions, to distinguish CIN involving immature metaplastic squamous epithelium from immature metaplastic squamous epithelium not involved by CIN and to distinguish high-grade CIN from benign mimics. p16INK4A stain is also found to be a valuable ancillary test in making the diagnosis of squamous dysplasia. Combined with the conventional hematoxylin and eosin stain, the p16INK4A staining may help to identify truly dysplastic foci in tissues which is particularly challenging, because of poor orientation or small biopsies, tissues that show severe inflammation or ulceration or keratinizing dysplasia. Other series indicated that p16 overexpression within malignant epithelium in the female genital tract should not be interpreted as synonymous with HPV-induced carcinogenesis. Data also indicated that p16 overexpression could occur in non-HPV-related cancers of the gynecological tract and that p16 overexpression suggested generic functional RB1 pathway abnormalities in cancer evolution. These preliminary results are promising; however, there is no corresponding report on colorectal carcinogenesis from normal-adenoma-carcinoma sequence. Therefore additional studies are needed to identify whether the p16INK4A as well as its binding protein, CDK4 has the similar prognostic value in predicting lesions that are likely to progress in colorectal neoplasm. We used the p16INK4A and CDK4 antibodies to stain reactive and dysplastic lesions in this series of colorectal normal-adenoma-carcinoma sequence and to investigate the possible role of p16 or CDK expression in the evolution of colorectal neoplasia.

MATERIALS AND METHODS

Samples

Forty-three cases of colorectal carcinoma with residual adenoma, in which adjacent normal mucosa was available in 42 of the cases, were randomly and retrospectively selected from the pathology files of St. Mark's Hospital, London and 31 cases of colorectal carcinoma were from the Department of Pathology, Chinese People's Liberation Army (PLA) General Hospital. Specimens obtained at surgery were routinely fixed in 10% neutral formalin and embedded in paraffin. The clinical stage was classified according to Dukes: 22 in Dukes' A, 22 in Dukes' B and 30 in Dukes' C, respectively. The histological grade of the tumors was determined according to WHO criteria as follows: 24 in grade I, well differentiated; 41 in grade II, moderately differentiated; and 9 in grade III, poorly differentiated.

Immunocytochemical analysis

Immunocytochemical staining for p16 and CDK4 was performed with a standard ABC method except that the pressure cooking procedure was used for antigen retrieval pretreatment^[15]. Serial sections were cut 4 µm thick and

dewaxed in xylene and rehydrated in a graded ethanol series. The sections were immersed in 3% hydrogen peroxide in methanol for 15 min to block endogenous peroxidase activity and rinsed in running water. Sections were then immersed in boiling 1 mmol/L EDTA-NaOH (pH 8.0) buffer in a pressure cooker. The pressure cooker was then sealed and brought to full pressure. The heating time was 2 min, beginning only when full pressure was reached. At 2 min the cooker was depressured and cooled under running water. The lid was then removed, and the hot buffer was flushed out with cold water from a running tap. The cooled sections were washed twice in PBS before immunohistochemical staining, then immersed in 0.05% avidin for 30 min to block any possible endogenous biotin exposed by heating. Prior to application of antibodies, the sections were incubated with 10% horse serum, for monoclonal antibodies and 10% goat serum, for polyclonal antibodies, respectively for 15 min to block non-specific binding. The primary monoclonal mouse antibody against human p16 protein (Pierce, USA) was produced, using a full-length recombinant bacterially produced GST-p16INK4 fusion protein as the immunogen, and could only be reactive to human tissue. The polyclonal rabbit antibody against human CDK4 protein (Santa Cruz Biotechnology, USA), was raised against the epitope corresponding to amino acids 282-303 mapping at the carboxy terminus of mouse CDK4 and is specific for CDK4 with no cross-reactivity with other cyclin dependent kinases in mouse, rat and human tissue. The antibodies were diluted 1:200 with 0.01 mol/L PBS (pH 7.2). After exposure to primary antibody the sections were allowed to react with the standard ABC method with VECTASTAIN Elite PK-6100 kit (Vector Laboratories, Inc. USA) as directed by the manufacturer. A previously known positive glioma was used as a positive control. The primary antibody was replaced by 0.01 mol/L PBS as a negative control. Normal colon mucosa tissue was used as a normal control.

Interpretation of p16 and CDK4 immunocytochemical staining

Immunostained sections were evaluated according to the localization of staining of positive tumor cells as follows: tumors were classified as cytoplasmic/nuclear (C/N) and cytoplasmic (C) pattern, respectively, when immunoreactivity was present in a large proportion of epithelial cells. When immunoreactivity was not visible, the result was classified as negative.

Statistical analysis

The correlation between variables was determined using the nonparametric test with SPSS10.0. A value of *P* less than 0.05 was accepted as statistically significant. The results are summarized in Table 1, Table 2, Table 3.

RESULTS

p16 subcellular expression in normal-adenoma-carcinoma sequence

Of the 42 normal epithelia examined, cytoplasmic/nuclear expression pattern was found in 40 (95.2%) and

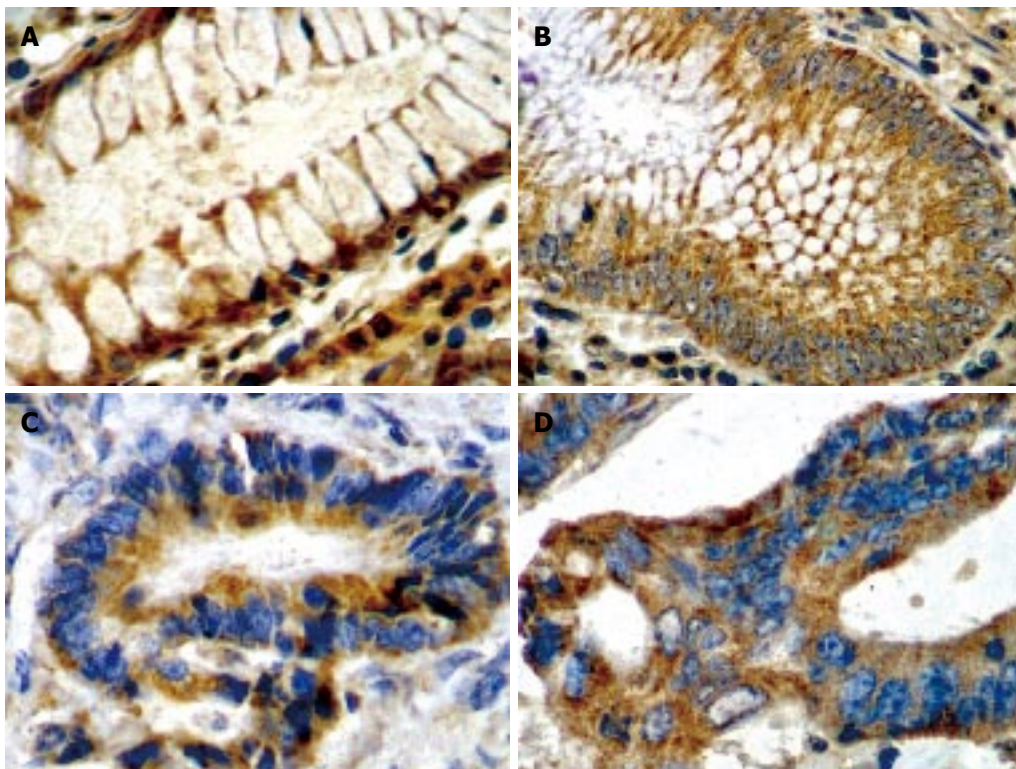


Figure 1 Subcellular expression of p16 and CDK4. **A:** Cytoplasmic/nuclear pattern of p16 in normal epithelia of colon mucosa ($\times 200$); **B:** Cytoplasmic pattern of p16 in adenomatous epithelia of adenoma ($\times 200$); **C:** Cytoplasmic pattern of p16 in colonic adenocarcinoma ($\times 400$); **D:** Cytoplasmic pattern of CDK4 in colonic adenocarcinoma. Original magnifications ($\times 400$).

Table 1 Relationship between the subcellular expression pattern of p16 or CDK4 and the histology of epithelia of normal-adenoma-carcinoma sequence

Histology	Subcellular expression pattern		
	Total	C/N	C
p16			
Normal	42	40	2
Adenoma	43	32	11
Carcinoma	73	27	46
$P < 0.001$			
CDK4			
Normal	38	29	9
Adenoma	43	10	33
Carcinoma	74	15	59
$P < 0.001$			

C/N: Cytoplasm/nucleus.

cytoplasmic only in 2 (4.8%) (Figure 1A). Of the 43 adenomas, 32 (74.4%) showed cytoplasmic/nuclear and 11 (25.6%) cytoplasmic only for p16 (Figure 1B). Of the 74 carcinomas arising from the adenomas, 27 (36.5%) showed cytoplasmic/nuclear and 46 (62.1%) cytoplasmic expressing pattern for p16 (Figure 1C). Only in one additional advanced carcinoma was the poorly differentiated signet cells negative. There was a significant difference between the patterns of p16 expression in any two positive types of epithelia of the normal-adenoma-carcinoma sequence ($P < 0.001$) (Table 1). In non-neoplastic mucosa adjacent to carcinoma, p16 expression was present weakly in the nucleus and moderately only in the cytoplasm around the nucleus. Most of the cytoplasm of goblet cells filled with mucus was negative. Expression for p16 was almost always observed strongly in the cytoplasm and sporadically weakly in the nuclei of cancer cells.

Table 2 Relationship between p16 subcellular expression pattern and clinicopathological features

Clinicopathological feature	n	p16 subcellular pattern	
		C/N	C
Dukes' stage			
A	22	8	14
B	22	6	16
C	29	13	16
Histological grade			
I	24	8	16
II	40	16	24
III	9	3	6
Total	73	27	46

C/N: Cytoplasm/nucleus. There was no significant correlation between p16 subcellular expression pattern and the Dukes' stage ($P = 0.441$) or histological grade ($P = 0.865$) of the carcinoma.

CDK4 subcellular expression in normal adenoma-carcinoma sequence

In non-neoplastic mucosa adjacent to carcinoma, CDK4 nuclear and cytoplasmic expression was slightly weaker than the p16 expression, but was otherwise identical. Of the 42 normal epithelia examined, CDK4 expression was cytoplasmic/nuclear in 29 (69.0%), cytoplasmic in 9 (21.4%), and negative in 4 (9.5%). Of the 43 adenomas, CDK4 expression was cytoplasmic/nuclear in 10 (23.3%) and cytoplasmic positive in 33 (76.7%). In 74 carcinomas, CDK4 staining was cytoplasmic/nuclear in 15 (20.3%) and cytoplasmic alone in 59 (79.7%) and like p16, almost always observed strongly in the cytoplasm (Figure 1D). There was a significant difference between the subcellular patterns of CDK4 expression in any two positive types of epithelia in the normal-adenoma-carcinoma sequence ($P < 0.001$) (Table 1).

Table 3 Relationship between CDK4 subcellular expression pattern and clinicopathological features

Clinicopathological feature	n	CDK4 subcellular pattern	
		C/N	C
Dukes' stage			
A	22	4	18
B	22	4	18
C	30	7	23
Histological grade			
I	24	4	20
II	41	10	31
III	9	1	8
Total	74	15	59

C/N: cytoplasm/nucleus. There was no significant correlation between CDK4 subcellular expression pattern and the Dukes' stage ($P = 0.844$) or histological grade ($P = 0.584$) of the carcinoma.

Correlation between p16 or CDK4 subcellular expression and Dukes' stage or histological grade in carcinomas

There was no significant correlation between p16 or CDK4 subcellular expression pattern and the Dukes' stage ($P = 0.441$; $P = 0.844$) or the histological grade ($P = 0.865$; $P = 0.584$) of the carcinoma (Tables 2 and 3).

DISCUSSION

Research on expression of p16 has made much progress in gynecologic pathology in recent years^[21,29-31]. p16 is found diffusely positive in most cervical carcinomas including squamous, glandular, and small cell type. In cervical glandular lesions, p16 is also useful to diagnose the lesions between adenocarcinoma *in situ*, which shows diffusely positive pattern and benign mimics, including tuboendometrial metaplasia and endometriosis, which usually shows p16-negative or focally positive pattern. Combined with other markers, p16 may be used to distinguish between a cervical adenocarcinoma (diffuse positivity) and an endometrioid-type endometrial adenocarcinoma (negative or focally positive). In some uterine serous carcinomas the staining is diffusely positive. In the vulva, p16 is positive in HPV-associated vulval intraepithelial neoplasia (VIN) but negative in VIN not associated with HPV. Similarly, HPV-associated invasive squamous carcinomas are p16-positive, whereas the more common non-HPV-associated neoplasms are largely negative or focally positive. In the uterus, p16 positivity is more common and widespread in leiomyosarcomas than in leiomyomas, and it may be a useful marker to diagnose problematic uterine smooth muscle neoplasms. Metastatic cervical adenocarcinomas in the ovary are usually diffusely p16-positive, and because these may closely mimic a primary ovarian endometrioid or mucinous adenocarcinoma, it may be a valuable diagnostic aid, although p16 expression in primary ovarian adenocarcinomas of these morphologic subtypes has not been widely investigated. Some ovarian serous carcinomas, similar to their uterine counterparts, are p16-positive. Darvishian *et al*^[32] reported that the sensitivity and specificity of p16 immunoreactivity in the detection of anal intraepithelial neoplasia or squamous cell carcinoma

were 72% and 71%, respectively. The presence of p16 immunoreactivity is a good predictor of dysplasia in anal specimens. Nilsson *et al*^[28] found that the expression of p16INK4a varied between the skin lesions including actinic keratosis, squamous cell carcinoma in situ and invasive squamous cell carcinoma, with weak and cytoplasmic p16INK4a expression and functional Rb in actinic keratosis. Strong nuclear and cytoplasmic p16INK4a expression was observed in all carcinomas in situ in parallel with a lack of Rb-phosphorylation but high proliferation, indicating a nonfunctional Rb. More interestingly, despite this disability of p16INK4a to inhibit proliferation there is an upregulation of cytoplasmic p16INK4a in infiltrative cells compared to tumor cells towards the tumor center, suggesting a potentially proliferation independent function for p16INK4a in infiltrative behavior. It has been reported that p16 overexpression is a potential early indicator of transformation^[30] and associated with poor clinical outcome in ovarian carcinoma^[31]. In addition, it was also reported that intensity of p16 expression may play an important role in clinicopathological features and prognosis of colorectal adenocarcinoma^[15-19]; However, there has been no subcellular localization analysis on colorectal epithelia of adenoma-carcinoma sequence compared with their normal counterpart.

In the current study, we aimed to clarify if p16 overexpression, with its binding protein, CDK4 overexpression has any role in colorectal neoplasia. Our result showed that p16 overexpression was almost always observed strongly in the cytoplasm and sporadically weakly in the nuclei of colorectal cancer cells and most carcinomas showed more cytoplasmic overexpression for p16 and CDK4 than the adenomas from which they were arising or the adjacent normal mucosa. Most normal or non-neoplastic epithelia showed more p16 and CDK4 expression in the cell nucleus than their adjacent adenomas and carcinomas. There was a significant difference between the subcellular expression pattern of p16 and CDK4 in normal-adenoma-carcinoma sequence epithelia ($P < 0.001$). The findings suggest that CDK4 may be activated as one of the initial events, which is followed by activation of the p16 gene, possibly as a feedback mechanism, with the effect of preventing G1/S transition through pRB phosphorylation by CDK4 overexpression. In normal colorectal epithelia there was much mucin in the cytoplasm. In the tumorigenesis, from normal epithelia to adenoma, and then to carcinoma, the mucin loss was observed gradually with the increase of p16 and CDK4 expression in the cytoplasm of epithelia. It is further suggested that cytoplasmic overexpressions for p16 and CDK4 may be involved in the mechanism for the loss of mucin and the transformation from normal mucosa to adenoma, and from adenoma to carcinoma. Cytoplasmic localization of p16 might be due to its binding with CDK4, forming a larger molecule, difficult to pass through the nuclear membrane. Loss of expression of p16 in the nucleus might suggest that there is a loss of function of p16 besides binding with CDK4, which negatively regulates the transcription of some important genes in tumorigenesis. p16 overexpression is known to occur in neoplastic cells as an indirect phenomenon of an aberrant

RB1 functional pathway, as is well known in carcinomas of the lower genital tract, in which high-risk types of HPV have caused inactivation of RB1. Some data^[33] also indicate that p16 overexpression may occur in non-HPV-related cancers of the gynecological tract and possibly indicate generic functional RB1 pathway abnormalities in cancer evolution in which p16 overexpression may not be caused by mutational silencing of the *Rb1* gene directly in the majority of cases, but rather the retinoblastoma pathway is rendered dysfunctional by some other mechanism. Unlike our previous report in intensity analysis^[15], there were no differences in p16 or CDK4 subcellular expression between Dukes' stages and histological grades in this research, indirectly suggesting that the subcellular expression of the two proteins may be significant in the evolution, but not in the progression, of colorectal carcinomas. Extensive research is needed to further clarify the mechanism.

In summary, our results support experimental evidence that interaction of expression of p16 and CDK4 plays an important role in the Rb/p16 pathway, and cytoplasmic overexpression of CDK4 and p16 as well as loss expression of p16 in nucleus might be important in the evolution of colorectal carcinoma from adenoma and, of adenoma from normal epithelia. Thus cytoplasmic overexpression of CDK4 and p16 may be a potentially early marker of transformation in colorectal carcinoma.

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Pyogenic liver abscess after choledochoduodenostomy for biliary obstruction caused by autoimmune pancreatitis

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Abstract

A 68-year-old man underwent cholecystectomy and choledochoduodenostomy for biliary obstruction and nephrectomy for a renal tumor. Based on clinical and histopathologic findings, autoimmune pancreatitis (AIP) was diagnosed. The renal tumor was diagnosed as a renal cell cancer. Steroid therapy was started and thereafter pancreatic inflammation improved. Five years after surgery, the patient was readmitted because of pyrexia in a preshock state. A *Klebsiella pneumoniae* liver abscess complicated by sepsis was diagnosed. The patient recovered with percutaneous abscess drainage and administration of intravenous antibiotics. Liver abscess recurred 1 mo later but was successfully treated with antibiotics. There has been little information on long-term outcomes of patients with AIP treated with surgery. To our knowledge, this is the second case of liver abscess after surgical treatment of AIP.

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Key words: Autoimmune pancreatitis; Biliary reconstruction; Liver abscess

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INTRODUCTION

The differentiation of autoimmune pancreatitis (AIP) from pancreatic cancer is sometimes difficult because these diseases share many clinical features^[1]. When pancreatic cancer cannot be ruled out, laparotomy or pancreatic resection may be performed for patients with AIP. To date, there has been little information on long-term outcomes of patients with AIP treated with surgery. Some reports have described recurrences of AIP and pancreatic insufficiency after surgery^[2-4]. However, there have been only two reports describing infections^[4,5]. To our knowledge, this is the second reported case of liver abscess after surgical treatment of AIP.

CASE REPORT

A 68-year-old man with bronchial asthma visited a neighborhood clinic. The patient began to feel abdominal fullness and decreased appetite in January 2000. After laboratory studies showed liver dysfunction and hyperglycemia, he was referred to our hospital in February 2000. He had no history of malignancy or autoimmune diseases. He was not a drinker. Physical examination showed no abnormal findings. Laboratory studies showed elevated liver enzymes and hyperbilirubinemia: total bilirubin was 34 mg/L (normal, 3-11) and direct bilirubin was 24 mg/L (normal, 0-6). Serum levels of pancreatic enzymes were as follows: amylase, 75 IU/L (normal, 50-200); lipase, 52 IU/L (normal, 8-46); and elastase I, 3900 ng/L (normal, 810-2960). A fasting plasma glucose test and an oral glucose tolerance test indicated diabetes mellitus. Serum levels of gammaglobulin (13.4 g/L) and IgG (14550 mg/L) were not elevated. Serum IgG4 was not examined. Antinuclear antibodies were negative. Serum levels of tumor markers, such as carcinoembryonic antigen and carbohydrate antigen 19-9, were normal. A dynamic computed tomography scan showed swelling of the gallbladder, dilatation of the common bile duct, and slight swelling of the pancreas head (3.5 cm × 3.0 cm) (Figure 1A and 1B). Furthermore, a 2.8-cm-diameter tumor was detected incidentally in the right kidney (Figure 1C). Endoscopic retrograde cholangiopancreatography showed that the main pancreatic duct in the head was irregularly narrowed and that the upstream duct was slightly



Figure 1 Dynamic computed tomography scan showing swelling of the gallbladder and slight swelling of the pancreas head (white arrow) (A). The pancreas body and tail are not swollen (B). A tumor is detected in the right kidney (C).



Figure 2 Endoscopic retrograde cholangiopancreatography showing short irregular narrowing of the main pancreatic duct in the head. The narrowing is less than one third of the length of the main pancreatic duct (white arrowheads).



Figure 3 Percutaneous transhepatic gallbladder drainage. Fluorography through the biliary drainage catheter shows obstruction of the distal common bile duct.

dilated (Figure 2). Contrast agent could not be injected into the common bile duct. Percutaneous transhepatic gallbladder drainage was performed for obstructive jaundice. Fluorography through the biliary drainage catheter showed obstruction in the distal common bile duct (Figure 3). After biliary drainage, the patient underwent laparotomy in March 2000, because pancreatic cancer could not be ruled out and because a malignant renal tumor was suspected. Because wedge biopsy of the pancreas head showed no malignancy, cholecystectomy and choledochoduodenostomy were performed for biliary obstruction. Simultaneously, nephrectomy was performed

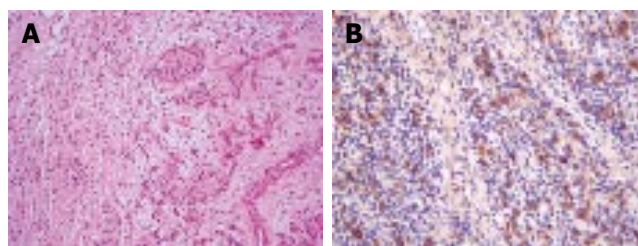


Figure 4 Wedge biopsy of the pancreas head reveals lymphoplasmacytic infiltration and interstitial fibrosis with acinar atrophy (HE x 100) (A). Immunohistologic study shows dense IgG4-positive plasma cell infiltration in peripancreatic lymph nodes (HE x 200) (B).

for the renal tumor. Further histopathologic examination of the pancreas specimen revealed lymphoplasmacytic infiltration and interstitial fibrosis with acinar atrophy (Figure 4A). An immunohistologic study performed 5 years after surgery showed dense IgG4-positive plasma cell infiltration in peripancreatic lymph nodes (Figure 4B). The renal tumor was diagnosed as a renal cell cancer (clear cell carcinoma, stage I).

Seven months after surgery, the patient complained of abdominal discomfort. Serum levels of pancreatic enzymes were markedly elevated: amylase, 621 IU/L; and lipase, 1082 IU/L. A serum level of carbohydrate antigen 19-9 was also elevated to 159.9 IU/mL (normal, 0-37). Dynamic computed tomography showed that swelling of the pancreas progressed from the head to the entire pancreas (Figure 5A and 5B). On the basis of clinical and histopathologic findings, AIP was diagnosed. Treatment with prednisolone of 5 mg per day was started. Thereafter, the symptom disappeared and serum levels of pancreatic enzymes were normalized. The swelling of the pancreas and irregular narrowing of the main pancreatic duct improved (Figure 6). Diabetes also improved.

In February 2005, 5 years after surgery, laboratory studies showed slightly elevated biliary enzymes, but the patient had no symptoms. In April 2005, the patient was admitted because of acute pyrexia in a preshock state. At that time, he had been continuing to take oral prednisolone, 5 mg per day. Laboratory studies showed elevated liver enzymes and an inflammatory response: white blood cell count, $18.9 \times 10^9/L$ (normal, $3.1-9.2 \times 10^9/L$) and C-reactive protein, 288 mg/L (normal, < 5). Blood culture was positive for *Klebsiella pneumoniae*. Ultrasonography and

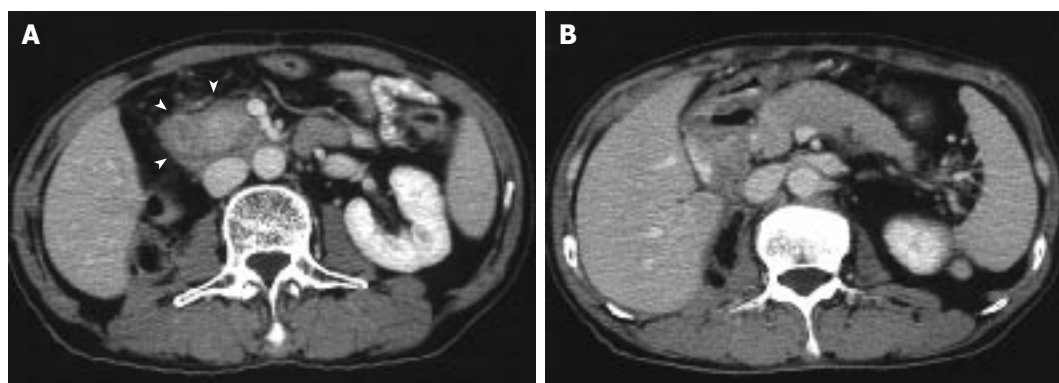


Figure 5 Dynamic computed tomography scan showing swelling of the pancreas head. A capsule-like low-density rim can be seen around the pancreas head (white arrowheads) (A). The pancreas body and tail are swollen (B).

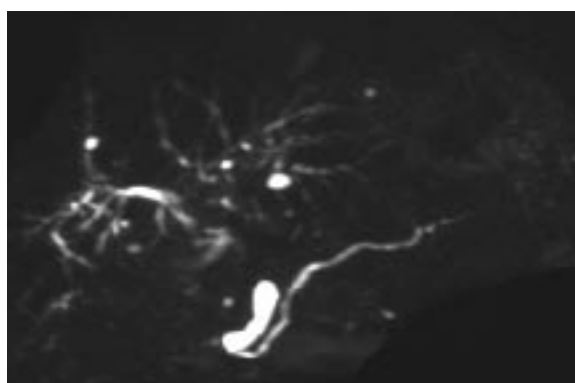


Figure 6 Magnetic resonance cholangiopancreatography performed 4 years after steroid therapy. The narrowing site is resolved in the main pancreatic duct.

computed tomography scan showed a multilocular liver abscess, 5.6 cm × 5.3 cm in size, in the right anteroinferior segment (Figure 7A and 7B). Vasopressor was administered for the preshock state, and percutaneous abscess drainage was performed for the abscess in combination with intravenous administration of cefoperazone/sulbactam at a dosage of 2 g per day. Prednisolone was stopped. A pus culture also showed *Klebsiella pneumoniae*. The patient was discharged after 2 wk of treatment; however, a liver abscess occurred in the left medial segment 1 mo later (Figure 7C), but was successfully treated with antibiotics. Hepatobiliary scintigraphy suggested no decrease of bile flow. The patient has been in good health since the second liver abscess was treated. AIP has not recurred irrespective of cessation of steroid therapy.

DISCUSSION

The clinical entity of AIP was established when Yoshida *et al*^[6] proposed the concept in 1995; however, a worldwide consensus on diagnostic criteria for AIP has not been achieved^[7]. Retrospectively, the present case did not satisfy the diagnostic criteria proposed by the Japan Pancreas Society^[8] at the onset of AIP. However, the results of histopathologic examinations^[9] and the clinical findings after surgery were compatible with AIP. Recent reports have described various AIP cases, such as the present one, in which pathologic changes are not found throughout the pancreas^[5,10-12]. These “focal” and “segmental” types

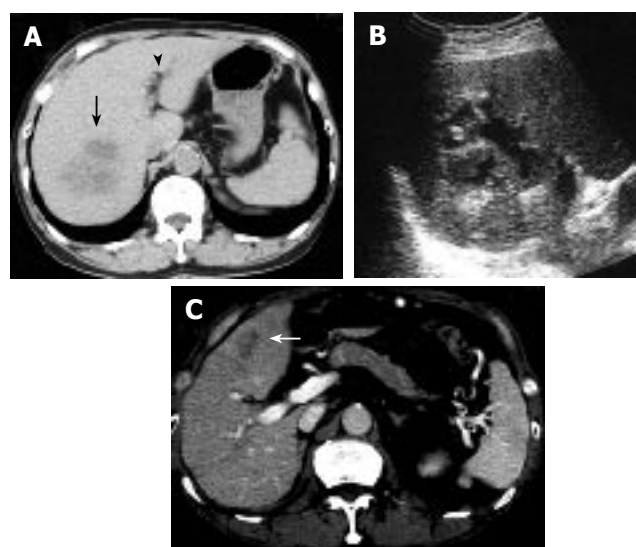


Figure 7 Computed tomography scan showing an abscess in the right anteroinferior segment of the liver (black arrow). Pneumobilia is detected (black arrowhead) (A). Ultrasonography shows partial liquefaction in the abscess (B). Computed tomography scan showing an abscess in the left medial segment of the liver (white arrow) (C). Note that the pancreas is not swollen.

of AIP have been considered to correspond to an early stage of AIP^[10-12]. To avoid unnecessary laparotomy or pancreatic resection, the establishment of definitive diagnostic criteria for AIP is urgent. If AIP is suspected but pancreatic cancer cannot be ruled out by imaging and laboratory studies, endoscopic ultrasonography-guided trucut biopsy may help establish a diagnosis of AIP^[13]. A short course of steroids can also be used to guide diagnosis^[7].

Horiuchi *et al*^[5] reported the first case of AIP associated with liver abscess. This is the second one. It should be noted that liver abscess developed several years after surgical treatment of AIP in both cases. The pathogenesis of liver abscess in the present case remains unclear. The route of infection seemed to be the biliary tract because other routes, such as the portal tract, were unlikely. AIP is often associated with sclerosing cholangitis^[14-16]. Biliary stenosis caused by sclerosing cholangitis or anastomotic stenosis can induce cholestasis, which may cause biliary infection and liver abscess. However, cholestasis was not observed in the present case, suggesting that cholestasis is not a prerequisite for liver abscess formation after surgery.

Furthermore, there have been few reports describing cases of liver abscess after choledochoduodenostomy. The sump syndrome, a complication of side-to-side choledochoduodenostomy, can cause secondary liver abscess^[17]; however, the present patient had undergone end-to-side choledochoduodenostomy before the development of liver abscess. There may be other factors contributing to the development of the patient's liver abscess in addition to duodeno-biliary reflux due to biliary reconstruction.

Putative predisposing factors for infection in patients with AIP are as follows. First, diabetes mellitus, which is often associated with AIP^[18], can compromise patients' defenses against infection. Recent reports have shown that diabetes is present in many patients with pyogenic liver abscess^[19,20]. Second, steroid therapy for AIP can impair immunity. In most patients with AIP and diabetes, steroid therapy improves diabetes; however, in some cases, steroid therapy induces or exacerbates diabetes^[18]. The present patient had been receiving long-term steroid therapy, which improved the diabetes but might have induced an immunocompromised state.

In conclusion, pyogenic liver abscess can occur as a late complication of biliary reconstruction in patients with AIP. Careful follow-up should be performed to allow early detection of such a serious complication in patients with AIP treated with surgery.

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Strongyloides hyper-infection causing life-threatening gastrointestinal bleeding

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Abstract

A 55-year old male patient was diagnosed with strongyloides hyper-infection with stool analysis and intestinal biopsy shortly after his chemotherapy for myeloma. He was commenced on albendazole anthelmintic therapy. After initiation of the treatment he suffered life-threatening gastrointestinal (GI) bleeding. Repeated endoscopies showed diffuse multi-focal intestinal bleeding. The patient required huge amounts of red blood cells and plasma transfusions and correction of haemostasis with recombinant activated factor VII. Abdominal aorto-angiography showed numerous micro-aneurysms ('berry aneurysms') in the superior and inferior mesenteric arteries' territories. While the biopsy taken prior to the treatment with albendazole did not show evidence of vasculitis, the biopsy taken after initiation of therapy revealed leukoclastic aggregations around the vessels. These findings suggest that, in addition to direct destruction of the mucosa, vasculitis could be an important additive factor causing the massive GI bleeding during the anthelmintic treatment. This might result from substances released by the worms that have been killed with anthelmintic therapy. Current guidelines advise steroids to be tapered and stopped in case of systematic parasitic infections as they might reduce immunity and precipitate parasitic hyper-infection. In our opinion, steroid therapy might be of value in the management of strongyloides hyper-infection related vasculitis, in addition to the anthelmintic treatment. Indeed, steroid therapy of vasculitis with other means of supportive care resulted in cessation of the bleeding and recovery of the patient.

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Key words: Myeloma; Immunosuppression; *Strongyloides*

INTRODUCTION

Chemotherapy of malignancies as well as immunosuppression of immunological disorders is frequently complicated with severe infections, including bacterial, viral, fungal or parasitic infections. We present here a case of life-threatening gastrointestinal bleeding related to strongyloides hyper-infection as a consequence of chemotherapy for myeloma.

CASE REPORT

A 55-year-old male patient was diagnosed with kappa light chain myeloma stage III-B based on the Salmon-Durei staging system. The patient started chemotherapy according to the VAD protocol^[1,2], consisting of vincristine, doxorubicin and high dose dexamethasone along with zoledronic acid every 4 wk. After he received three full courses of chemotherapy the patient was admitted to the hospital for generalized fatigue, and fever with signs and symptoms of chest infection. Laboratory investigations showed haemoglobin of 105 g/L, white blood cell count of 5.5×10^9 /L (neutrophils 0.84, lymphocytes 0.11, monocytes 0.02, eosinophils 0.00, basophils 0.00, bands 0.03), and platelet counts of 1.91×10^{11} /L. His blood chemistry and coagulation profile were within normal limits except a low albumin level of 29 g/L (normal: 35-48 g/L). Chest X-ray showed no obvious abnormality. After obtaining blood and urine cultures ceftazidime therapy was started with an assumption of respiratory tract infection. For the following two days the patient kept a spiking fever which was associated with progressive pulmonary symptoms (dry cough and dyspnea). His oxygen saturation dropped to 90% in room air, and a moderate eosinophilia was observed: neutrophils 0.76, lymphocytes 0.12, monocytes 0.02, eosinophils 0.08, basophils 0.00, bands 0.02. On physical examination he had bilateral wheezes and basal crackles.

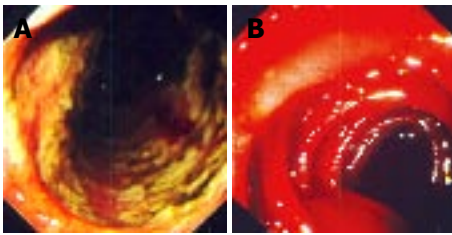


Figure 1 Endoscopic findings. **A:** Extensive confluent necrotic ulcerations of the duodenal mucosa due to chemotherapy related *S. stercoralis* helminthic hyper-infection. **B:** Enteroscopic presentation of diffuse mucosal bleeding during anthelmintic therapy of *S. stercoralis* hyper-infection, aggravated potentially by toxic/inflammatory compounds from destroyed parasites.

The chest X-ray was suspicious of hilar infiltrates at that time. A full cardiac assessment was done, and both ECG and echocardiography were within normal limits.

Subsequent spiral CT scan was not confirmatory for potential opportunistic chest infection, and the antibiotics however were changed to high dose septrin and levofloxacin intravenously. Serology tests for *Legionella*, *Mycoplasma*, hepatitis A, B, C, cytomegalovirus and Epstein-Barr virus were all negative. The blood culture grew staphylococcus and for that reason the portacath of the patient was removed and vancomycin was given. In spite of these managements, the patient's general condition did not improve. He presented soon new symptoms of dysphagia, odynophagia and vomitus, associated with major metabolic disturbances (sodium 117 mmol/L [N: 136-145 mmol/L], albumin corrected calcium 1.84 mmol/L [N: 2.2-2.6 mmol/L], and albumin 21 g/L [N: 35-48 g/L]), all quite resistant to treatment. Many options in the differential diagnosis were considered including inappropriate anti-diuretic hormone secretion (urine and plasma osmolality test and CT of the brain requested) and other endocrine dysfunction (PTH, TSH, free T₄, T₃, cortisol levels investigated). All of these investigations showed no hormonal abnormality.

Possibilities of gastrointestinal (GI) losses were also considered (exudative enteropathies, motility disorders, etc.). Plain abdominal X-ray, barium swallow and esophago-gastro-duodenoscopy (OGD) were completed. OGD showed diffuse confluent necrotic ulcerations in the stomach and the duodenum (Figure 1A). Biopsies were taken and intravenous omeprazole was started. A few days later the patient complained of epigastric pain accompanied with bilious and feculent vomitus. On examination, his abdomen was distended with decreased bowel sounds. A decubitus abdominal X-ray showed multiple fluid levels consistent with paralytic ileus. By this time, stool parasitology (Figure 2) as well as histopathology from both the duodenum (Figure 3A) and the stomach confirmed *Strongyloides stercoralis* helminthic infection with heavily infiltrated intestinal mucosa, but without histology criteria of vasculitis (Figure 3B). The patient received albendazole therapy (400 mg twice daily). Two days later, the patient developed massive GI bleeding with hypovolemic shock and major drop in the haemoglobin (40 g/L) requiring intensive care unit monitoring. His platelet count and coagulation profile were both normal at the first presentation of GI bleeding. Repeat OGD, magnetic resonance imaging study of the

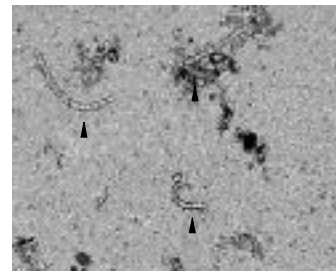


Figure 2 *S. stercoralis* larvae in the stool.

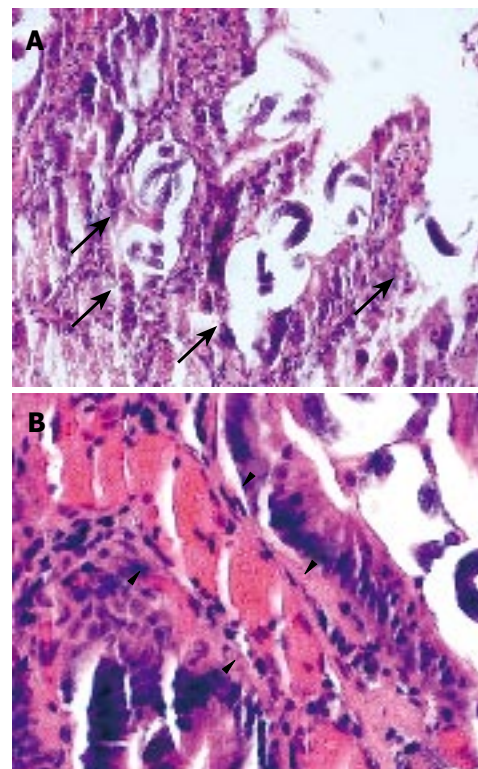


Figure 3 **A:** Intraluminal and intramucosal *S. stercoralis* larvae in the duodenum during the exudative enteropathy hyper-infection phase (arrows). Scale: 200 μ m. **B:** No intramural or peri-vascular inflammatory cell clusters are recognised during this early period of infection. Scale: 50 μ m.

abdomen and pelvis, radio-labelled red blood cell (RBC) nuclear investigations failed to localize a specific site of the bleeding. Colonoscopy was inconclusive due to heavy continuous flow of feculent content mixed with fresh blood from the small intestine. Abdominal aorta angiography showed numerous micro-aneurysms in the superior and inferior mesenteric arteries' territories with segmental strictures on the small intestine, consistent with vasculitis (Figure 4).

With repeated episodes of massive blood loss, during an overnight call an explorative laparotomy was decided on to localize a potentially resectable particular region of the intestine-as a desperate, life-saving procedure. During the procedure an intra-operative intestinoscopy was performed showing diffuse small intestinal bleeding (Figure 1B). No intestinal resection was done. However, as an additional proof of vasculitis at this point, intestinal biopsy revealed leukoclastic aggregations around the vessels of the mucosa (Figure 5). Screening for connective tissue diseases including immunoglobulin levels, rheumatoid factor, anti-

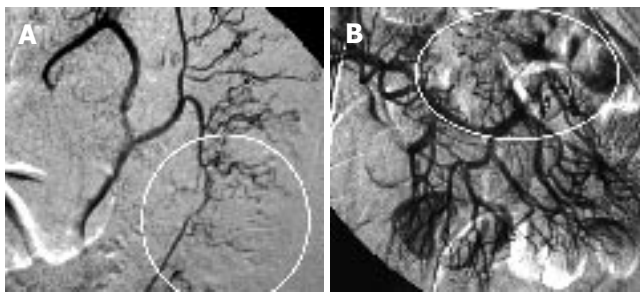


Figure 4 A: Segmental vasospasms (circled area). B: "berry-type" microaneurysms (circled area) in the mesenteric superior and inferior arteries' territory are both suggestive of active vasculitis during the anthelmintic therapy of *S. stercoralis* hyper-infection.

nuclear, anti-smooth muscle, and anti double-strand DNA antibodies was not suggestive of systemic autoimmune disorder. The patient was managed with huge amounts of blood transfusion, fresh frozen plasma, tranexamic acid (Cyklokaprone®), somatostatin, and, to correct secondary coagulopathy, with recombinant factor VIIa (Novoseven®). In addition, he was given antibiotics, anthelmintic drugs (albendazole and ivermectin) and steroids (dexamethasone). He required more than 120 units of RBC transfusion and 16 doses of Novoseven®. After 10 d of intensive support the bleeding gradually subsided. Stool analysis became repeatedly negative for strongyloides. An endoscopy was carried out 20 d later and showed complete recovery of the mucosa on review as well as on histopathology.

DISCUSSION

Strongyloidiasis is a worldwide infection, caused by the nematode *Strongyloides stercoralis* helminth^[3]. This thread-worm spreads in poorly sanitized areas from moist soil. It is endemic in the tropical Asia, Africa, Latin America, Southern US and Eastern Europe. In Southeast US its prevalence is as high as 4%^[4,5]. Chronic infection with *S. stercoralis* is usually asymptomatic, but it may manifest with symptoms such as episodic creeping urticaria, epigastric crampy abdominal pain, or diarrhea. Myeloma, as well as its chemotherapy can interfere with the immune competence of the patients. In addition to more frequent bacterial, viral and fungal infections, disseminated parasitosis may develop in such situations. There is remarkable absence of cellular immune response to these invaders specifically eosinophilia^[4]. In case of *Strongyloides stercoralis*, steroids may not only affect the host's cellular immunity, but also mimic an endogenous parasitic-derived regulatory hormone^[5,6]. Strongyloides were noticed to produce more eggs in the presence of exogenous steroids. Due to immunosuppressant therapy, there is a larger proportion of the rhabditiform larvae which mature into the filariform larvae within the host. This leads to a greater larval load and disseminated hyper-infection^[5,7]. Secondary bacterial infections are also frequent complications of dissemination phase. Pulmonary symptoms of our patient at his post-chemotherapy admission to the hospital are likely to be related to pulmonary migration of the larvae during the dissemination phase.

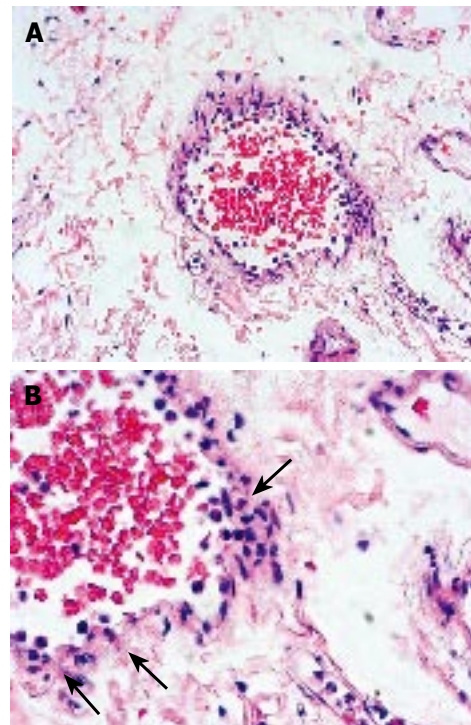


Figure 5 Perivascular granulocytic infiltration in the mucosa during the anthelmintic therapy of *S. stercoralis* hyper-infection is also suggestive of active vasculitis. Scale: A: 200 µm; B: 50 µm.

In hyper-infection syndrome, complete disruption of the GI mucosa, ulcerations, paralytic ileus with exudative enteropathy as well as massive GI bleeding may also occur due to the direct invasion of the larvae. Profound diarrhea, malabsorption with consequent hypo-albuminemia and electrolyte disturbances were all consistent with hyper-infection related enteropathy in our patient. On the other hand, effective anthelmintic treatment in hyper-infected patients can lead to mass-destruction of intraluminal and intramural larvae and to release of huge amounts of different toxic inflammatory and vaso-active compounds^[7,8]. Angiography and histopathology findings after initiation of albendazole therapy were consistent with active vasculitis in our patient, caused potentially by such compounds. For that reason we decided to maintain steroid therapy instead of rapid tapering and discontinuation of it, as advised by some experts. In our opinion, vasculitis due to helminth-derived inflammatory factors might contribute to mucosal disruption and massive GI bleeding. Steroids might reduce inflammation in such situation. Anthelmintic treatment and supportive management with transfusions of blood products and parenteral nutrition were also maintained until no more larvae could be detected in the stool. Finally, we have to emphasize the heroic multi-disciplinary efforts that led to a complete recovery of this 55 year old patient with strongyloides hyper-infection related life-threatening gastrointestinal bleeding.

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Right colon and liver hemangiomatosis: A case report and a review of the literature

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Abstract

Cavernous hemangiomatosis of the colon and liver in a 38-year-old woman presenting with a history of cramp like abdominal pain and a mass in the right iliac fossa are presented. Abdominal ultrasonography and computed tomography demonstrated multiple liver hemangiomas as well as a noncystic lesion in the right iliac fossa. Operative findings were suggestive of diffuse hemangiomatosis of the right colon and an extensive right hemicolectomy was performed. A review of the literature is presented, considering current diagnostic and therapeutic methods.

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Key words: Hemangiomatosis; Right Colon; Liver

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INTRODUCTION

Hemangiomas constitute 7% of all benign vascular tumors and are characterized by increased numbers of normal or abnormal vessels filled with blood and are usually localized; however, when they involve a large number of organs in the body the situation is called angiomatosis or hemangiomatosis^[1]. Although a variety of histological and clinical types of hemangiomas exist, capillary and cavernous subtypes are most frequently encountered. Cavernous hemangiomatosis of the colon is uncommon. However, the case reported in this study presents the hemangiomatosis of

the colon and the liver, a situation rarely reported.

CASE REPORT

A 38-year-old female patient presented with a 2-mo history of cramp like abdominal pain and radiating ipsilateral lumbar pain, without any rectal passage of blood. The physical examination did not reveal any specific signs. The abdominal ultrasound revealed multiple liver lesions measuring 10 mm × 23 mm each and the computed tomography demonstrated a large non-cystic lesion in the right iliac fossa, as well as multiple small liver lesions with heterogeneous enhancement (Figure 1). Operative findings were suggestive of right colon diffuse hemangiomatosis, innumerable mesenteric and multiple small liver hemangiomas. An extensive right hemicolectomy and end-to-end ileo-transverse anastomosis was carried out.

A specimen of a right hemicolectomy was sent to the Pathology Department, presenting grossly as a reddish-hemorrhagic tumor at the ileo-colic valve level, measuring 4 cm × 5 cm (Figure 2). The tumor had a sponge-like consistency and infiltrated the entire wall thickness extending in to the surrounding fatty tissue. The mesocolic and mesenteric fat, as well as the greater omentum, presented multiple hemorrhagic nodules measuring 0.6-1 cm in greater diameter. The specimen was subjected to routine procedures and multiple histological sections, stained with hematoxylin-eosin, were studied microscopically (Figures 3 and 4). The diagnosis of diffuse cavernous hemangiomatosis of the colonic and enteric wall with concomitant lymphangiectatic lesions of the submucosa and infiltration of the mesocolic and mesenteric fat and the greater omentum was decided.

DISCUSSION

Hemangiomatosis of the colon is a rare benign disease arising from the submucosal vascular network. According to the literature these lesions originate from embryonic sequestrations of mesodermal tissue^[2]. Histologically, hemangiomas are distinct from telangiectasias and angiodysplasias and approximately 80% of colonic hemangiomas are of the cavernous subtype. Capillary hemangiomas are usually solitary and produce no symptoms, while cavernous hemangiomas composed of large thin-walled vascular channels, have no capsule, involve the rectosigmoid region in up to 70% of the cases and present with rectal bleeding (60%-90%), anemia (43%), obstruction (17%) and rarely with platelet sequestration, although approximately 10%



Figure 1 Abdominal computed tomography demonstrating liver hemangiomas.



Figure 2 Macroscopic appearance of the surgical specimen, showing the colon hemangiomatosis.

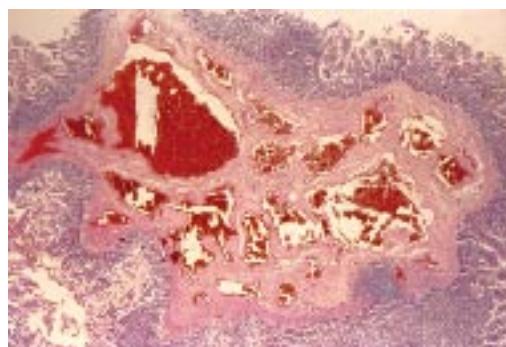


Figure 3 Histological section showing enteric wall mucosa and angiomatous lesions through the muscular layer of the colonic wall (HE x 25).

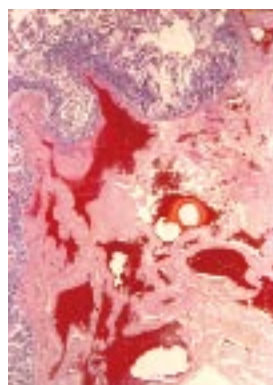


Figure 4 Histological section of colonic wall showing multiple hyperemic angiomatous spaces in the muscular layer (HE x 25).

of patients remain asymptomatic^[3]. A rare case of intussusception in a 39-year old male due to a capillary hemangioma of the right colon has been reported in the literature as a rare complication of colon hemangiomas^[4]. The average age of the patient at presentation is 12 years, equally affecting males and females^[2]. A case report of a 3-mo old infant presenting with ascites, anemia, minimal rectal bleeding and thrombocytopenia due to several hundred small cavernous hemangiomas of the colon and peritoneal surfaces who underwent subtotal colectomy^[5] and a 9-mo old child with an isolated colonic cavernous hemangioma of the hepatic flexure^[6], as well as a 3-wk old boy with diffuse hemangiomatosis of the small bowel^[7] are some of the youngest cases reported from the review of the literature. Up to 80% of patients undergo an unnecessary surgical procedure before diagnosis is made^[8], with an average delay time of 19 years elapsed between initial symptoms and diagnosis. Hemangiomatosis affecting the right colon is rather a rare case and from the review of the English literature fewer than 10 cases have been reported since 1950^[4,6,9-12]. Liver and bladder involvement seems to be unusual as well^[13,14]. The presence of phleboliths on plain abdominal radiographs is an important diagnostic clue, seen in 26%-50% of adult patients, indicating a sequential process of thrombosis in the tumor caused by perivascular inflammation and stasis of blood flow^[15]. In our patient no phleboliths were demonstrated on plain abdominal radiographs. Barium enema reveals non specific polypoid or multilobular masses that collapse with air insufflation. Endoscopically, a bluish, soft, submucosal polypoid mass consistent with widened varices usually indicates the pres-

ence of a hemangioma. Mesenteric angiography shows hypervascularity and delayed venous pooling associated with cavernous hemangiomas. Abdominal CT scans can give useful information about the size of the lesion and the extension to adjacent organs. Recently, three dimensional CT colonography was used to gain a more complete knowledge of the characteristics and distribution of lesions throughout the colon^[9]. Complete surgical resection is the definitive therapy, although (1) a pedunculated polypoid hemangioma (2) less or equal to 2.5 cm, with (3) an endoscopic ultrasonography demonstrating that the depth is limited in the submucosal layer are the three major criteria for endoscopic polypectomy^[16]. In conclusion, a high suspicion index is required when a young patient presents with abdominal pain, painless rectal bleeding, phleboliths in plain radiographs or heterogeneous masses in CT, in order to approach a definite diagnosis before planning any therapeutic intervention.

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American College of Gastroenterology
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Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
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Easl 2006 - the 41st annual
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Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
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Foundation of the Czech Society of Hepatology
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www.czech-hepatology.cz/phm2006

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 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

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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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- 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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Identification of differentially expressed genes in mouse hepatocarcinoma ascites cell line with low potential of lymphogenous metastasis

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Abstract

AIM: To identify genes differentially expressed in mouse hepatocarcinoma ascites cell line with low potential of lymphogenous metastasis.

METHODS: A subtracted cDNA library of mouse hepatocarcinoma cell line with low potential of lymphogenous metastasis Hca-P and its synogenetic cell line Hca-F with high metastatic potential was constructed by suppression subtracted hybridization (SSH) method. The screened clones of the subtracted library were sequenced and GenBank homology search was performed.

RESULTS: Fifteen differentially expressed cDNA fragments of Hca-P were obtained with 3 novels.

CONCLUSION: Tumor metastasis is an incident involving multiple genes. SSH is a useful technique to detect differentially expressed genes and an effective method to clone novel genes.

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Key words: Suppression subtracted hybridization; Liver neoplasm; Metastasis suppression genes

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INTRODUCTION

Tumor metastasis is an incident involving multiple genes. However, the number of metastasis related genes available nowadays is very limited to elucidate the puzzling process of metastasis. Therefore, more attentions have been paid to screen candidate genes responsible for metastasis by high throughput technique. Hca-P and Hca-F are a pair of synogenetic mouse hepatocarcinoma ascites cell lines, possessing a specific potential of lymphogenous metastasis when inoculated subcutaneously into 615 mice, Hca-P showing a low metastatic potential ($< 30\%$), while Hca-F showing a high potential ($> 80\%$)^[1]. In the current study, we employed suppressive subtracted hybridization (SSH) technique to identify differentially expressed genes specific for Hca-P in an effort to obtain candidate genes related to lymphogenous metastasis of hepatocarcinoma in mice.

MATERIALS AND METHODS

Hca-F and Hca-P have been established and maintained by our laboratory^[1]; inbred 615-mice were provided by the experimental animal center of our university.

Determination of lymph node metastatic rates of Hca-P and Hca-F

Sixty inbred 615-mice were randomly divided into 2 groups. The Hca-P and Hca-F tumor cell lines were inoculated at 2×10^6 tumor cells of approximately 0.1 mL cell suspension into each mouse subcutaneously in each group. The mice were decapitated on the 28th day post-inoculation. The implanted tumor and the regional lymph nodes were removed and paraffin sections of tissues were HE stained and examined under microscope. The lymph node metastatic rates of Hca-F and Hca-P tumor cells were calculated.

Construction of a subtracted cDNA library by SSH

Preparation of total RNA and mRNA: Isolation of total RNA was performed by TRIZOLTM(GIBCOBRL) and that of mRNA was carried out according to the protocol of oligotex mRNA spin column purification kit (Qiagen). The quantity and integrity of mRNA were detected by ultraviolet spectrometer and by electrophoresis on a denaturing formaldehyde agarose stained by EB. mRNA of Hca-P served as tester and mRNA of Hca-F

as driver. SSH was performed between tester and driver by a PCR selectTM cDNA subtraction kit and 50 × PCR enzyme kit (Clontech, Heidelberg, Germany) following the instructions of the manufacturer.

dscDNA synthesis and digestion with *Rsa* I : Briefly, 2 µg aliquots of each of poly (A⁺) mRNA from the tester and the pooled driver were subjected to dscDNA synthesis. Thereafter, they were purified by passing through Chroma spin-400 columns (Clontech, USA). Each purified dscDNA was digested with *Rsa* I .

ligation to adaptor 1 and 2R: The tester cDNAs were subdivided into 2 equal groups and then ligated to adaptor 1 and 2R in separate ligation reactions. Ligation efficiency analysis was performed by amplifying ligation products with G3PDH 3' primer/PCR primer 1 and G3PDH 3' primer/G3PDH 5' primer, respectively, and their intensity was compared.

Subtractive hybridization: Subtractive hybridization was performed by annealing an excess of driver cDNAs with each sample of adaptor-ligated tester cDNAs. The cDNAs were heat-denatured and incubated at 68°C for 8 h. After the first hybridization, the 2 samples were mixed together and hybridized again with freshly heat-denatured driver cDNAs for 20 h at 68°C. Two rounds of hybridization would generate a normalized population of tester-specific cDNAs with different adaptors at each end. After filling in the ends, 2 rounds of PCR amplification were performed to enrich desired cDNAs containing both adaptors by exponential amplification of these products^[2]. The optimized cycles for the first and second PCRs were 27 and 13 respectively to increase representation and reduce redundancy of subtracted cDNA libraries.

Analysis of subtractive efficiency: Secondary PCR products were used as templates for PCR amplification of human G3PDH for 18, 23, 28 and 33 cycles respectively to assure subtraction efficiency. PCR products were run on 1.8% agarose gel.

Ligation of the subtracted library into a TA vector

Products of the secondary PCR reactions were cloned into a pT Adv vector (Clontech) and the resultant ligation products were then transformed into DH5α *E. coli* competent cells. The bacteria were subsequently grown in 800 µL of liquid Luria-Bertani medium and allowed to incubate for 45 min at 37°C with shaking at 150 rpm. Thereafter, the cells were plated onto agar plates containing ampicillin (50 µg/mL), 5-bromo-4-chloro-3-indoly-b-D-galactoside (x-gal; 20 µg/cm²) and iso-plopr-b-D-thiogalactoside (IPTG; 12.1 µg/cm²) and incubated overnight at 37°C. Individual recombinant white clones were picked and grown in single line pattern onto Luria-Bertani agar solid medium containing ampicillin and allowed to incubate at 37°C for 6-7 h before single clone was picked from single-line pattern agar medium and allowed to grow in Luria-Bertani liquid medium containing ampicillin overnight at 37°C with shaking at 150 rpm.

Identification of the subtracted clones

Plasmids of candidate positive clones from subtracted cDNA library were isolated and amplified by PCR with

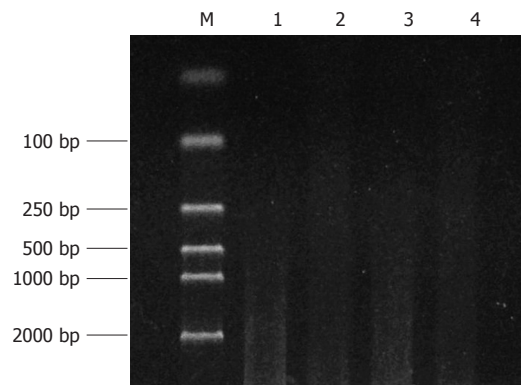


Figure 1 The effect of *Rsa* I digestion. Lane1, 3: cDNA of Hca-F and Hca-P cells; Lane 2, 4: cDNA of Hca-F and Hca-P cells after *Rsa* I digestion; M: DNA Marker DL2000.

nested primer 1 and primer 2. Meanwhile the product of PCR was detected by agarose gel electrophoresis.

Sequencing and BLAST homology search

Randomly screened 14 positive clones from the subtracted cDNA library were sequenced by T7/SP6 chain termination reaction in TaKaRa (DaLian, China). Nucleic acid homology searches were subsequently performed at the National Center of Biotechnology Information (National Institutes of Health, Bethesda, Md., NCBI).

RESULTS

Determination of lymph node metastatic rates of Hca-P and Hca-F

Implanted tumors of both Hca-P tumor-bearing mice and Hca-F tumor-bearing mice were palpable on 7th day post-inoculation. On the 28th day post-inoculation, 10% Hca-P cells bearing mice developed metastatic regional lymph nodes (3/30), while 80% Hca-F cells bearing mice developed metastatic regional lymph nodes (24/30).

Total RNA and mRNA analysis

The RNA samples electrophoresed on 1% agarose/EB gel exhibited 2 typical bands, corresponding to ribosomal 28s and 18s RNA, respectively, with a ratio of intensities > 2:1 and 1.9, ideal A260/A28 ratios of both samples obtained, indicating high integrity and purification of the total RNA we obtained. mRNA samples appeared as a smear with weak ribosomal RNA band: a high-quality mRNA was purified.

Rsa I digestion

Both the digested cDNA and undigested cDNA usually presented as smears. However, their patterns were different. The digested cDNA fragments became shorter after *Rsa* I digestion (Figure 1).

Ligation efficiency analysis

Intensity of the PCR product amplified using one gene-specific primer (G3PDH 3' primer) and PCR primer 1 was 25% more than that of PCR product amplified using two

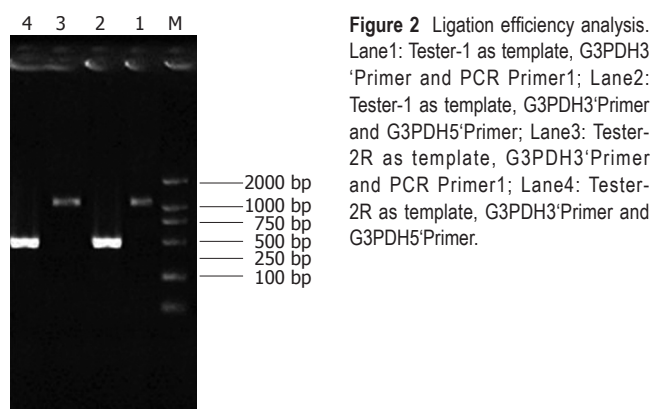


Figure 2 Ligation efficiency analysis. Lane1: Tester-1 as template, G3PDH3'Primer and PCR Primer1; Lane2: Tester-1 as template, G3PDH3'Primer and G3PDH5'Primer; Lane3: Tester-2R as template, G3PDH3'Primer and PCR Primer1; Lane4: Tester-2R as template, G3PDH3'Primer and G3PDH5'Primer.

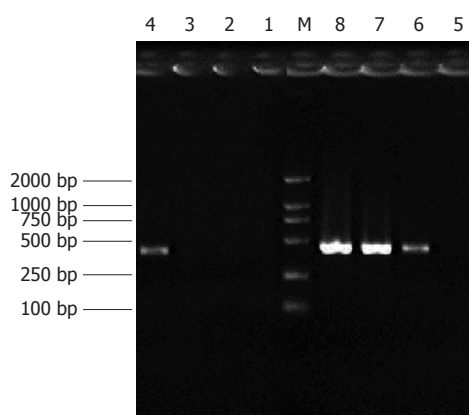


Figure 4 Analysis of subtraction effect. PCR was performed on subtracted(Lane1-4) or unsubtracted(Lane5-8) secondary PCR product with G3PDH 5'Primer and 3' primer. Lane1, 5: 20 cycles, Lane 2, 6: 25 cycles, Lane 3, 7: 30 cycles, Lane 4, 8: 35 cycles. M: DNA Marker DL2000.

gene-specific primers (G3PDH 3' primer and 5' primer). Ligation efficiency was > 25%, ensuring enough tester cDNA in the following hybridization (Figure 2).

Construction of subtracted cDNA library by SSH

PCR products of the subtracted and unsubtracted usually looked like smears with or without discrete bands. However, the patterns between them were different (Figure 3).

Analysis of subtractive efficiency

Subtraction efficiency analysis showed the effectively reduced amount of non-differentially expressed genes. In unsubtracted cDNA libraries, housekeeping gene G3PDH PCR products were visible after 23 cycles of amplification and became saturated after 23-28 cycles. However, subtracted libraries required 33 cycles for G3PDH to be detected (Figure 4).

Differential screening of subtracted cDNA libraries

The subtracted cDNA libraries were composed of 995 positive clones, of which 200 clones were randomly picked up and plasmids of the candidate positive clones were isolated and amplified by PCR with nested primer 1 and primer 2. As a result, 189 positive clones showed PCR products of a size of 300-1000 bp (Figure 5).

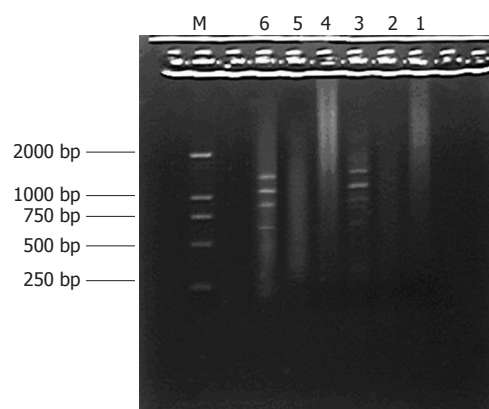


Figure 3 The results of secondary PCR amplification. Lane 1-3: Product of primary PCR amplification, Lane 4: secondary PCR amplification product of PCR control cDNA, Lane5: secondary PCR amplification product of unsubtracted cDNA, Lane6: secondary PCR amplification product of subtracted cDNA, M: DNA Marker DL2000.

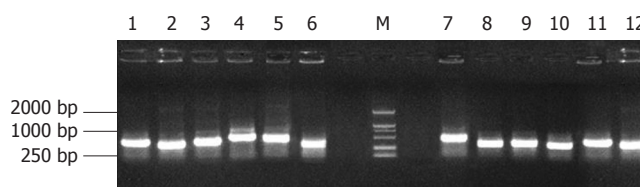


Figure 5 The results of clone PCR amplification, There was a average insert size of 300-1000 bp.

Sequencing and homology search

Fifteen screened clones randomly selected were sequenced and homology search (<http://www.ncbi.nlm.nih.gov/BLAST/>) revealed 8 known genes and 4 expressed sequence tags (ESTs). Three cDNAs showed no homology and presumably represented novel genes (Table 1).

DISCUSSION

Tumor metastasis, as the leading cause of tumor related death, is a process involving multiple genes and their products. Elucidation of the gene expression profiles specific for tumor cells with different potential of metastasis might help in the understanding of the molecular mechanisms of metastasis. As one of the high throughput screening techniques, SSH technique has two distinct advantages: (1) it boasts a high subtraction efficiency; (2) it harbors an equalized representation of differentially expressed sequences which can separate effectively both high and low copy expressed genes mainly because of normalization^[2]. von Stein *et al*^[3] found about 94% positive rate in their research. Thus they considered confirmation of differentially expressed genes by Northern blot analysis for each clone obtained was probably unnecessary.

For a long time, studies have focused on the angiogenesis of tumors, but the roles of lymphatic vessels in tumor growth and metastasis were neglected. However, it is well known that lymphatic metastasis is mainly responsible for the spread of epithelial malignant tumors,

Table 1 Homologue searching of the sequenced cDNA fragments from SSH library

Clone serial number	Size (bp)	Sequence identity
1-3	508	Mus musculus Telomere repeat binding factors TRF1
3-5	543	Mus musculus Telomere repeat binding factors TRF2,
9-6	489	Mus musculus maspin
15-4	335	mouse chromosome 3 clone RP 6-126M1
11-7	386	mouse 7 d embryo whole body cDNA RIKEN full-length enrich library, clone 2210102k3
16-3	503	mouse 5 d liver cells cDNA RIKENfull -length library enriched library, clone E330462F4
10-1	549	mouse chromosome 17 clone RP 26-122M3
13-7	502	mouse chromosome 4 clone RP13-110N10
9-8	340	EST-mouse
6-6	411	EST-mouse
11-2	390	EST-mouse
20-9	399	EST-mouse
23-3	470	unknown
17-6	486	unknown
12-3	344	unknown

and is closely related to the prognosis of patients.

Hca-P and Hca-F are a pair of syngeneic mouse hepatocarcinoma ascites cell lines presenting a specific potential of lymphogenous metastasis with a significant difference in their potential of metastasis^[1]. Candidate genes involved in lymphogenous metastasis are supposed to be among the differentially expressed genes.

Using Hca-P as tester, Hca-F as driver, we employed SSH technique to identify differentially expressed genes specific for Hca-P(low metastatic potential) so as to obtain candidate suppressor genes of lymphogenous metastasis. Fifteen screened clones randomly selected were sequenced and homology search revealed 8 known genes as TRF₁, TRF₂(telomere repeat binding factor 1, 2); maspin; mouse 7 days embryo whole body cDNA, RIKEN full-length enriched library clone 2210102k3; mouse 5 days liver cells cDNA, RIKEN full-length enriched library, clone E330462F4; mouse chromosome 3 clone RP 6-126M1; mouse chromosome 17 clone RP 26-122M3 and mouse chromosome 4 clone RP13-110N10. Studies showed TRF₁ and TRF₂ play important roles in genome stabilization^[4-11] and are down-regulated in some malignant cell lines and tumor tissues^[12-18]. In hematopoietic carcinogenesis, gene expression of telomerase suppressors such as TRF and TIN2, is decreased. mRNA encoding TRF₁ and TRF₂ when gastric cancer becomes more deeply invaded, is significantly decreased, indicating a negative association with tumor progression. Of the 8 known fragments, one showed high homology to mouse Maspin gene. Serving as one of the few p53-targeted genes involved in tumor invasion and metastasis, Maspin, a member of the serpin family, has been reported to suppress metastasis and angiogenesis in breast and prostate cancers, and is closely correlated with their clinical manifestations^[19-31]. It indicates that SSH in our study is capable of enriching metastasis related genes.

Another 5 known fragments were attributed to embryo

genes. Embryo genes AFP and CEA are overexpressed in hepatocarcinoma and other malignant tumors, indicating a possible association between embryo development and tumor. Embryo genes were also found in our previously established SSH library which contains candidate tumor metastasis genes^[32]. These data showed that up-regulated expression of embryo genes during metastasis is not a casual event. Their roles in tumor metastasis need to be clarified. Moreover, 4 cDNA fragments demonstrated homology with 4 ESTs-mouse and 3 cDNA fragments showed no homology and presumably represented novel genes^[33].

In summary, the findings of our study suggest that the lymphatic invasiveness of tumor cells is determined by multiple genes and co-factors with complex cellular signal pathways. Further functional study of the candidate novel genes might provide clues to molecular mechanism of tumor metastasis.

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Precise role of *H pylori* in duodenal ulceration

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Abstract

The facts that *H pylori* infection is commoner in duodenal ulcer (DU) patients than in the normal population, and that eradication results in most cases being cured, have led to the belief that it causes DU. However, early cases of DU are less likely than established ones to be infected. *H pylori*-negative cases are usually ascribed to specific associated factors such as non-steroidal anti-inflammatory drugs (NSAIDs), Crohn's disease, and hypergastrinaemia, but even after excluding these, several *H pylori*-negative cases remain and are particularly common in areas of low prevalence of *H pylori* infection. Moreover, this incidence of *H pylori* negative DU is not associated with a fall in overall DU prevalence when compared with countries with a higher *H pylori* prevalence. In countries with a high *H pylori* prevalence there are regional differences in DU prevalence, but no evidence of an overall higher prevalence of DU than in countries with a low *H pylori* prevalence. There is no evidence that virulence factors are predictive of clinical outcome. After healing following eradication of *H pylori* infection DU can still recur. Medical or surgical measures to reduce acid output can lead to long-term healing despite persistence of *H pylori* infection. Up to half of cases of acute DU perforation are *H pylori* negative. These findings lead to the conclusion that *H pylori* infection does not itself cause DU, but leads to resistance to healing, i.e., chronicity. This conclusion is shown not to be incompatible with the universally high prevalence of DU compared with controls.

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Key words: Duodenal ulceration; *H pylori* infection; Not causal; Delays healing

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INTRODUCTION

The award of the Nobel Prize to Warren and Marshall for the discovery of *H pylori*^[1] was rightly acclaimed by the medical profession, because the eradication of the organism turns a chronic, relapsing disease into one that in most (but not all) cases can be readily cured. The prevalence of infection with the organism is greater in patients with, than in subjects free of, duodenal ulcer; the organism is present in most (but not all) cases of the disease; and its removal results in most (though not all) cases being cured without relapse. The inference usually drawn from this combination of events is that *H pylori* actually causes duodenal ulcer.

In that case, why do most individuals infected with the organism not develop the disease? There has been considerable work done exploring the concept that the presence or absence of virulence factors explains this anomaly, but in a recent publication^[2] we explain why we do not find this hypothesis convincing.

This unsatisfactory situation, the facts that most patients with infection do not have a duodenal ulcer, that, geographically, duodenal ulcer prevalence is not related to the prevalence of *H pylori*, and many other anomalies, has been highlighted in several review articles, such as *H pylori*: the African Enigma^[3]. The enigma is two-fold: firstly, that DU prevalence is not higher in countries with a higher prevalence of *H pylori* infection, secondly, that within these countries, despite an uniformly high *H pylori* prevalence, regional differences in DU prevalence are found, that as described later, are related to diet but not to smoking, genetic or other factors.

Our continuing search for an explanation of the anomalies led us to review all the papers that provide evidence about the following: (1) Is *H pylori* infection present at the onset of DU? (2) Is there a dose-response relationship between *H pylori* and DU? (3) Does recurrence or non-recurrence of DU after successful treatment correlate with *H pylori*-status? (4) Can we deduce anything of importance from the prevalence and distribution of *H pylori*-negative DU?

The present paper analyses the evidence of the literature on these subjects and we show how the higher prevalence of *H pylori* in DU than in the normal population and some of the other anomalies can be explained by a self-consistent theory, but only by relinquishing the belief

that *H pylori* causes DU.

To the best of our knowledge, no paper has been published that contradicts statements in this review. We shall be obliged to readers for letting us know about any exceptions that we have missed.

Is *H pylori* infection present at the onset of DU?

One is entitled to expect that the cause always precedes its effect. The problem with duodenal ulceration is that the patient usually presents to the physician some time after the symptoms start: it is unlikely that evidence of infection has been sought before the symptoms began. It occurred to us to examine the *H pylori*-status of patients with duodenal ulcer in relation to the length of history before the initial biopsy that established the diagnosis of DU. Assuming the organism was the cause of the ulcer, we expected the infection to be manifest at least as often in the first six months as it was more than six months after symptoms started. We studied 37 patients: to our surprise, 5 whose history was of less than six months were *H pylori*-negative, the remaining 32, all with a length of history greater than six months, were *H pylori*-positive. The odds against this happening by chance were (Fisher's exact test) greater than 1000 to 1^[4]. It looked as though the DU was causing the infection, rather than the other way about.

Searching the literature at that time (2001), we could find only one paper that gave figures for infection status, distinguished by different lengths of history^[5]. Repeating the search in 2005, we were only able to find one further paper with the appropriate data^[6]. Both these papers showed a higher infection rate later compared with earlier in the ulcer disease.

There are two reports^[7,8] which suggest that pre-existing *H pylori* infection predisposes to DU based on seropositivity. The first paper refers to young inductees into the Israeli army and the second to an older population in Hawaii. In the first paper it is noteworthy that 7 of the 29 reported DU cases were *H pylori*-negative at the time of diagnosis. However, seropositivity does not mean that infection is present. After eradication, it can take 5-10 years for seropositive cases to become seronegative. Many children serorevert after childhood infection and in adults the conversion/reversion rates per annum vary between 0.5% and 1%. An approach along these lines therefore lacks scientific reliability.

Dose-response relationship?

The smallest dose is zero. Many patients develop a DU in the absence of infection with *H pylori*. This fact is usually explained by invoking 'special' causes of DU such as Zollinger-Ellison syndrome and non-steroidal anti-inflammatory agents (NSAIDs) (see later), but such causes are not always apparent.

Before the *H pylori* era, excess gastric hydrochloric acid was the favoured aetiology of DU. 'No acid, no ulcer' has been dogma since Schwartz^[9], and there has been no contrary evidence. However, acid is 'statistically' greater than the normal range in only about 15% of (patients with) DU, the rest having acid in the normal range^[10], albeit with a tendency to be greater than in the normal

population.

In many countries there is no information about the actual prevalence of DU in the overall population, and available information is based on figures obtained from hospital statistics or small population surveys. From information that is available, there is no evidence that there is a higher overall prevalence of DU in those countries where there is a higher prevalence of *H pylori* infection than in other countries. There is evidence from India, China and Africa, however, of differences in DU prevalence between areas known to have a high prevalence of *H pylori* infection^[3,11-18] these differences being related to the staple diets of the regions^[15-24] rather than to smoking, genetic or other factors such as duodenal gastric metaplasia. There is evidence that different foods contain agents that are either ulcerogenic or ulceroprotective^[24-27].

In developed countries, where *H pylori* prevalence is about 35% overall, DUs are about 70% *H pylori*-positive^[6,28-38]; in developing countries, *H pylori* prevalence is about 70% overall, and DUs are *H pylori*-positive in about 90% of patients^[16,28,29,39-50]. Therefore in all countries *H pylori*-positivity is greater in DU than in non-DU. With reference to further discussion, it is notable that in areas of low *H pylori* prevalence there is a high prevalence of *H pylori*-negative, non-NSAID, ulcers^[4,6,16,28,29,34,38,39,51-63].

As mentioned above there is no evidence that the prevalence of DU is any greater in countries with a high prevalence of *H pylori* than in those with a low prevalence (this finding in itself argues against *H pylori* being the prime cause of DU). The prevalence of DU in London is about 11%^[10]. Applying this figure to other developed countries, from the figures quoted previously in every 100 of the population, 35 are *H pylori*-positive and 65 *H pylori*-negative. There are 11 patients with DU, of whom perhaps only one is *H pylori*-negative, so only 10 of the 35 *H pylori*-positive subjects get DU. The remaining 25 (25/35, 71%) do not. A similar argument in the developing countries (again assuming an 11% prevalence) yields an estimate that only 10 of the 70 *H pylori*-positive subjects get DU. The remaining 60 of the 70 (86%) do not. The belief that *H pylori* is the prime cause of DU demands an explanation of why in 71%-86% of individuals it does *not* produce a DU.

Weight of infection can be measured by use of the breath test, but no-one has suggested that the greater the weight of infection, the greater is the risk of developing DU. However, there is excellent evidence that the risk of developing DU increases with the rate at which the subject secretes gastric juice when maximally stimulated with intravenous histamine^[10]. The *H pylori*-lobby suggests that we cannot demonstrate a dose-relationship of DU with *H pylori* because only some strains of the organism possess virulence factors (of which many have been reported). It is beyond the scope of this paper to go into that topic, but there is considerable evidence that virulence factors have no relationship to clinical outcome^[2].

Does recurrence of DU correspond with *H pylori*-status?

The answer is, apparently not very well. Ulcers heal with effective medical suppression of acid without eradication

of *H pylori*^[64,65], and after surgical procedures ulcers remain healed despite persistent *H pylori* infection^[66-75].

When *H pylori* was discovered and the first results came in about the effect of its extirpation, it was claimed that removing *H pylori* 'cured' the disease and that relapses never occurred. As time passed that picture had to be modified: there is no doubt that relapses are much less frequent and many patients have no further trouble, but there is increasing evidence of a significant recurrence rate after eradication of *H pylori* despite lack of recurrence of the infection. Excluding subjects taking NSAIDs, 9 papers, involving 2928 DU patients in whom *H pylori* had been eradicated as proven by multiple tests, reported recurrent ulceration in 182 (6.1%) over a period up to 5 years^[76-84]. One meta-analysis by Laine of 7 trials subjected to strict criteria reports a recurrence rate of 20% within 6 mo^[81]. Interestingly, a recurrence rate of 6.6% (571/8693) up to 2 years is given in 12 papers (including 6 meta-analyses)^[76,84-96] involving 8693 cases of DU, not excluding NSAIDs, in whom *H pylori* had been eradicated. These recurrence rates, with and without NSAIDs, are virtually identical ($P = 0.4883$). In other words, recurrence after eradication of *H pylori* cannot be attributed to NSAIDs. The use of multiple tests for *H pylori* reduced the risk that we are dealing here with difficulty in demonstrating the presence of *H pylori* after eradication.

What does *H pylori*-negative DU tell us?

The phenomenon of *H pylori*-negative DU is an argument against a blanket role for *H pylori* as a "cause preceding effect". As stated above, this prevalence is greater in countries with a low, compared with countries with a high prevalence of the organism, even after excluding DU-associated factors such as Crohn's disease and the taking of NSAIDs. There are 20 such reports^[4,6,28,29,34,38,39,51-63] from countries with a low prevalence of *H pylori* infection giving a mean of 14.4% (829/5745) of *H pylori*-negative DU and 5 reports^[47,96-99] with a mean of 3.9% (52/1325), $P < 0.0001$ from countries with a high prevalence.

Three papers^[100-102] suggest that despite the low *H pylori* prevalence in a population with an increased prevalence of *H pylori*-negative DU, there is no decrease in overall DU prevalence. If *H pylori* were the primary cause of DU then one would expect a lower prevalence of DU.

We can offer one supplementary consideration. Perforation of duodenal ulceration might reasonably be expected to signify an especially large secretion of gastric hydrochloric acid. In this context it is interesting that perforation does not seem to be associated with *H pylori*. In 4 reports about patients operated on for perforated duodenal ulcer *H pylori* prevalence was significantly less than in uncomplicated duodenal ulceration^[103-106] (in 2^[103,104] they were indistinguishable from normal controls). The only dissenting evidence was from a report by Matsukara^[107].

INTERPRETATION

How are we to interpret the undoubted relationships between DU, *H pylori* and gastric acid? The present

majority view is that *H pylori* causes DU, not that DU causes *H pylori*. The favourable evidence for the former inference is the greater proportion of *H pylori*-positive cases in DU compared with non-DU subjects, and the fact that clearing the organisms converts the clinical course of DU from chronic relapsing to (mostly) stable healing. However, the second of these points is not proof of initial causation, merely of an interference with healing leading to chronicity of the ulceration.

If *H pylori* is not the initial cause of DU?

If we reject *H pylori* as the cause of DU, how can we explain the greater proportion of *H pylori* in DU compared with non-DU subjects? *H pylori* can only live within a relatively narrow band of pH. Both highly acid and highly alkaline conditions kill the organism^[108,109]. For example, in pernicious anaemia the patient is usually *H pylori*-positive in the early stages (acid production is reduced but still abundant) and then becomes *H pylori*-negative in the later stages (when all acid production has ceased and the stomach is exposed to alkaline reflux from the duodenum)^[110,111].

These facts suggest that some patients who develop a DU may well have so much acid that they are *H pylori*-negative. When treated with acid suppression for their early symptoms, the gastric acidity may fall enough to encourage infection with the organism. At this stage they are investigated and found to be *H pylori*-positive. Strange as it may at first seem, we are postulating that one interpretation of the link between *H pylori* and DU in cases that are initially *H pylori*-negative is that DU (*via* its treatment) causes the infection. This would explain the greater prevalence of *H pylori* in the subjects with DU as an outcome of treatment with acid-suppressing drugs. If the likelihood of a first infection increases with the presence of virulence factors in the organism - as seems reasonable - this explains why the virulence factors are more prevalent in the DU than in the non-DU subjects^[2].

In developing countries with a high prevalence of *H pylori* infection, and where people do not have access to acid-suppressing drugs and only come to hospital with long-standing chronic conditions, there is another possible explanation. With a high *H pylori* prevalence of 70% it follows that 30% of DU patients initially would be *H pylori* negative and 85% of them will not be hypersecretors of acid (See 'Dose Relationship' above). As a result of continued exposure to the high prevalence of *H pylori* a number of these may become infected, resulting in their ulceration becoming unremitting and chronic, and causing them to seek medical help. The result again would be a higher prevalence of *H pylori* infection in those diagnosed with duodenal ulceration.

In addition, the known lability of *H pylori* infection could result in some *H pylori* positive DUs healing as a result of spontaneous disappearance of the infection, leaving a preponderance of DU cases with persisting infection, thus resulting in a higher prevalence of infection in the DU population.

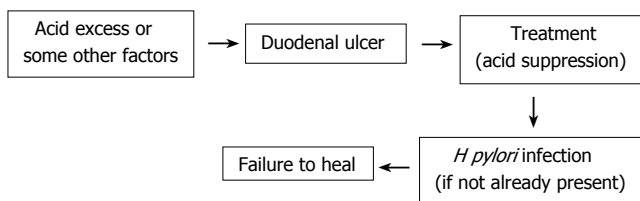
It is possible to calculate the relationship between the fraction of established duodenal ulcer patients who are

H. pylori-positive, the fraction of the whole population who are *H. pylori*-positive, and the fraction, X, of those initially *H. pylori*-negative duodenal ulcer patients who become infected so as to produce the observed increase in established DU patients. We have performed these calculations on data from 18 reports from countries with a high prevalence^[16,28,29,35,39-50,112] and 13 reports from countries with a low prevalence of *H. pylori* infection^[5,28-38,113]. The values of X were 0.6 in the developing countries, 0.58 in the developed. The congruence between these two estimates, while certainly not proof of our hypothesis, at least suggests that our hypothesis should not be rejected out of hand. Details of the derivation of the formula and the statistical interpretations are given in the Appendix.

There remain two important problems. (1) Most patients with DU have gastric secretion within the range of normal (though the chance of getting DU does increase with the rate of secretion). There must be some other factor to explain why some get an ulcer and others do not. Our work^[114] demonstrated that this factor was unlikely to be *H. pylori*. We favour the idea that the causative agent nevertheless involves interference with wound-healing. NSAIDs can interfere with the healing of *H. pylori*-negative ulcer, so they may be responsible for part of the problem, but there is almost certainly more to discover; (2) We still do not know how *H. pylori* (which can only live in gastric, not in true duodenal mucosa), makes the ulcer difficult to heal. The presence of colonised gastric mucosa within the duodenum might be a factor. *H. pylori* infection inhibits healing of wounded duodenal epithelium in vitro due to vacA^[115]. Another factor might be the effect that the organism has of increasing sub-maximally (gastrin-) stimulated gastric juice^[116]. While the mechanism is in doubt, the relationship is clear and is the fundamental reason why the discovery of *H. pylori* is of such enormous importance for the treatment of duodenal ulcer.

CONCLUSION

Our present view is that the relationship between duodenal ulcer, *H. pylori* and gastric acid secretion is most likely to be:



APPENDIX

Fraction (= X) of *H. pylori* -ve duodenal ulcers becoming *H. pylori* + ve, possibly as a result of antacid treatment

$$X = \frac{\% \text{ Hp+ve DU minus } \% \text{ Hp+ve NUD or Controls}}{\% \text{ Hp -ve NUD or Controls}}$$

Hypothesis

Whether or not a subject is *H. pylori*-positive (+ve) or *H. pylori*-negative (-ve) makes no difference to the likelihood that s/he will develop a duodenal ulcer (DU).

The diagnosis of most subjects with a DU is only made after the patient has already been treated with agents that reduce gastric acid secretion.

Reduction in gastric secretion is likely to increase the chance that a -ve patient becomes +ve.

When first diagnosed, most DU patients will include some who were +ve before they developed DU and others who had been -ve but became positive during their initial treatment and before diagnosis.

Calculation

Let

P = population

U = fraction having DU

F = fraction of population *H. pylori* +ve
(so 1-F = fraction negative)

Then

Confirmed DU (say after 6/12)

$$= U \times P$$

However, the confirmed DU is made up of two moieties, one originally +ve, the other originally negative.

The originally positive DU number

$$= F \times U \times P$$

The originally negative DU number

$$= (1-F) \times U \times P$$

Let a fraction X of the originally negative DU be infected as a result of acid-suppression. Then after 6/12 these will number

$$= (1-F) \times U \times P \times X$$

Therefore the observed positive DUs can be expressed as $(F \times U \times P) + X[(1-F) \times U \times P]$.

Therefore, the observed proportion of DUs who are positive is given by

$$\{(F \times U \times P) + X(1-F) \times U \times P\} / (U \times P)$$

or, dividing by $U \times P$,

$$F + X(1-F)$$

Therefore, observed +ve DU/(total DU) = $\{F + X(1-F)\}$

$$= F + X - FX$$

Therefore, $X(1-F) = [\text{observed DU+ve}/\text{total DU}] - F$

And $X = \{[\text{observed DU+ve}/\text{total DU}] - F\} / (1-F)$

Analysis of the figures quoted

High prevalence countries: Mean Hp+ DUs = 89.395; (SD 7.602, SE 1.700). Mean Hp+ NUD = 68.350 (SD 12.759, SE 2.853). Mean X = 60.565 (SD 26.466, SE 5.918).

Low prevalence countries: Mean Hp+DU = 70.221; (SD 14.319, SE 3.183). Mean Hp + NUD = 33.286; (SD 16.014, SE 4.280). Mean X = 58.279; (SD 17.773, SE 4.750)

Difference between high prevalence and low prevalence countries: for Hp + DU, $P = 0.0002$ (highly significant); for X, $P = 0.4109$ definitely non-significant.

This hypothesis suggests that in all countries there is much the same chance of originally *H. pylori*-negative becoming infected as the ulcer progresses. This fact provides circumstantial evidence, though certainly not proof, of our hypothesis.

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EDITORIAL

Mannose-binding lectin and maladies of the bowel and liver

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Abstract

Mannose-binding lectin (MBL) is a pattern-recognition molecule that binds to characteristic carbohydrate motifs present on the surface of many different pathogens. MBL binding stimulates the immune system *via* the lectin pathway of complement activation. In certain clinical situations, often characterized by pre-existing immune compromise, MBL deficiency increases the risk of infectious and other disease-specific complications. Many of the key pathogenic processes inherent to common gastroenterological diseases, such as infection, immunological damage, and carcinogenesis, have been linked to MBL. This editorial reviews the biology of MBL, outlines key disease associations to document the breadth of influence of MBL, and finally, highlights the relevance of MBL to both gastroenterological health and disease.

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Key words: Mannose-binding lectin; Collectins; Innate immunity; Polymorphism; Infection

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INTRODUCTION

Mannose-binding lectin (MBL) is an important component

of the innate immune system. MBL is primarily produced by the liver, circulates throughout the body, and is able to recognize a wide array of common pathogens through repeating carbohydrate sequences present on microbial surfaces. MBL binding of pathogens initiates complement activation via the lectin pathway. There have been a large number of studies addressing the influence of MBL deficiency on infection, autoimmunity, and carcinogenesis, all critical processes in the pathogenesis of gastrointestinal disease. Genetically determined MBL deficiency increases the risk and manifestations of a wide range of diseases, particularly when the immune system is already compromised. This editorial provides an introduction to the structure, function and regulation of MBL and explores its clinical relevance, placing it in the context of common medical and, in particular, gastrointestinal conditions.

THE BIOLOGY OF MANNOSE-BINDING LECTIN

The MBL2 Gene

The capacity of MBL to recognize and eradicate pathogens is extremely variable. Within any given population there are individuals that have varying functional levels of circulating MBL. The relative sufficiency of MBL function for any given individual is largely determined by polymorphisms within the *MBL2* gene, on chromosome 10. Three missense mutations within the first exon of *MBL2* significantly effect MBL function (codon 54 'B', codon 57 'C', and codon 52 'D') (Figure 1). These coding mutations are collectively designated 'O'; and the wild-type, sufficient allele is represented by 'A'. An Australian study of healthy blood donors found that the prevalence of *MBL2* wild-type coding genotype *A/A* was 57.6%, coding mutation heterozygosity (*A/O*) was 34.8% (*A/D* 11%, *A/B* 19.9%, *A/C* 3.8%), and coding mutation homozygosity (*O/O*) was 7.6% (*B/B* 2.1%, *B/C* 2.1%, *B/D* 2.5%, *D/D* 0.9%)^[1]. These frequencies are consistent with other Caucasoid populations^[2-4]. In Asian communities, the most common mutation is also the 'B' allele, but the 'D' allele is virtually absent^[5].

Further variability of MBL function is due, at least in part, to other polymorphisms within the promoter (position -550, G to C substitution, alleles 'H' and 'L' and position -221, G to C substitution, alleles 'X' and 'Y'); and 5'-untranslated (position +4, C to T, alleles 'P' and 'Q') regions of the gene (Figure 1). When inherited in the context of a normal coding allele (*A*), the promoter region haplotypes

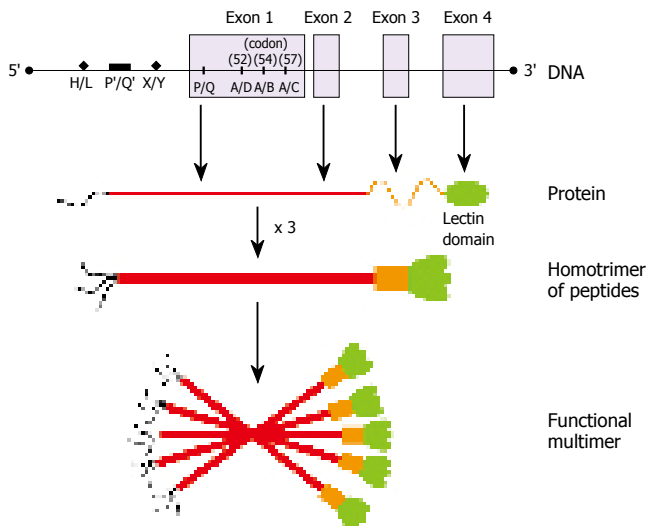


Figure 1 Schematic representation of the four exons of the *MBL2* gene, with the important polymorphisms identified. The peptides self associate into a homotrimer (structural subunit). Each peptide contains a lectin domain (green) to bind the specific, microbial carbohydrate motifs. Functional MBL circulates in higher-order multimers.

HY, *LY*, and *LX* are associated with high, intermediate, and low serum MBL concentrations, respectively. The genotypes *O/O*, *A/O* and *LXA/LXA*, are all associated with low antigenic and functional levels of MBL (compared to *A/A*). The *O/O* genotype is correlated with the most extreme MBL deficit (Figure 2). Low levels of MBL associated with the common polymorphic variants appear to result from impaired oligomerization of the MBL triple helix (see below) into functional higher order multimers^[6], as well as increased susceptibility to degradation by metalloproteinases^[7].

The MBL Protein

The basic structural subunit of MBL is a homotrimer of MBL peptides, entwined in a triple helix (Figure 1). Each peptide contains a lectin domain to bind the specific oligosaccharide motifs present on the surface of many different microorganisms^[8]. Functional MBL circulates as a higher-order multimer (tetramers, pentamers and hexamers) of the basic MBL subunit. This oligomerization allows high-affinity interaction between MBL and the microorganism. Binding of MBL to pathogens causes a conformational change in the MBL multimer, and activation of associated molecules, the MBL-associated serine proteases (MASPs), that initiate the lectin-complement pathway.

The lectin-complement pathway

The enzymatic cascade of complement activation is a vital aspect of innate immunity. Complement-derived opsonization also provides an effective means of articulation with adaptive immunity through subsequent phagocytosis and antigen processing. The classical complement pathway is initiated by the binding of the C1 complex (C1q, r and s) to bound antibody on pathogen surfaces and the alternative pathway by binding of C3b to hydroxyl or amino groups on cell-surface molecules, as a result of spontaneous C3 turnover^[9]. The lectin-

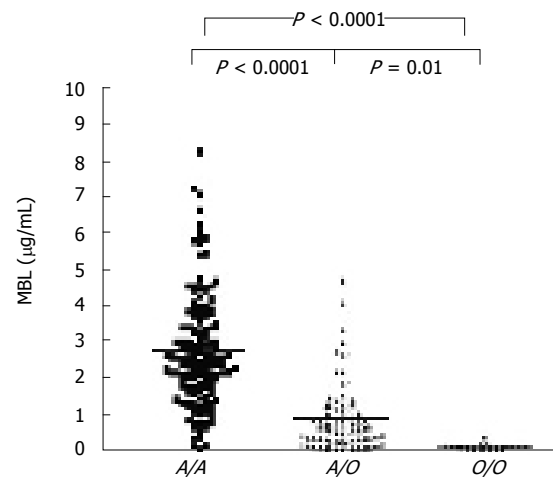


Figure 2 Distribution of plasma MBL levels within a normal population, stratified by *MBL2* genotype (Figure reproduced from Worthley *et al.*^[82] with permission). The *O/O* genotype is associated with the most extreme deficit in circulating MBL level (shown) and activity.

complement pathway is the third arm of complement activation. Higher order MBL multimers circulate in a functional complex with three serine proteases MASP-1, MASP-2, MASP-3 and one non-protease molecule, Map19^[8]. This complex is analogous to the C1 complex that initiates the classical complement pathway, except that MBL binds to pathogens independently of antibody. Once activated, MASP-2, like its classical-pathway counterpart C1s, cleaves C4 to C4b, producing C4b2a, the C3 convertase. Subsequent production of C3b, also a key opsonin, generates the C5 convertase, which in turn produces the chemoattractant C5a, and, through C5b, the formation of the membrane-attack complex, C5b-C9 (Figure 3).

Recently, an additional mechanism of lectin-mediated complement activation, which bypasses the classical pathway proteins, has been described. Selander *et al.*^[10] demonstrated that an MBL-dependent alternative pathway mediated C3 deposition in C2 deficient serum. This bypass pathway may be of particular significance in the presence of complement deficiencies^[11].

THE CLINICAL RELEVANCE OF MANNOSE-BINDING LECTIN

MBL binds a broad range of bacteria, viruses, fungi and protozoa (Table 1). Its affinity for Gram-negative and Gram-positive bacteria is mediated through cell surface components, such as lipopolysaccharide (endotoxin) and lipoteichoic acid, respectively. MBL deficiency increases *in vivo* susceptibility to many common bacterial infections, including *Neisseria meningitidis*^[12], *Streptococcus pneumoniae*^[13], and *Staphylococcus aureus*^[14]. MBL deficiency may also increase the risk of several viral infections and some of the most compelling data in this area have been conducted in viral hepatitis, discussed below.

The balance of evidence suggests that MBL deficiency is most relevant when immunity is already compromised as a consequence of immunological immaturity, for example

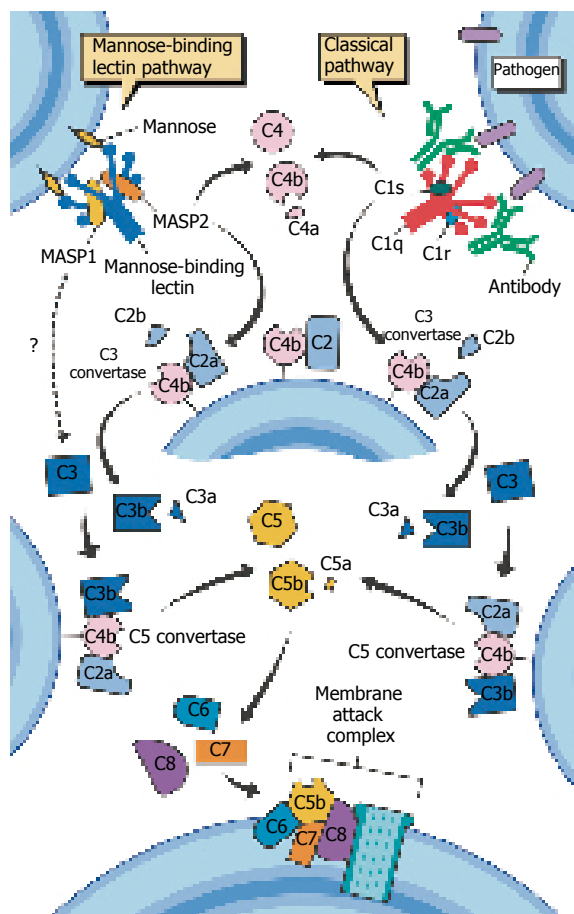


Figure 3 The lectin and classical complement pathways (Figure reproduced from Worthley *et al*^[82] with permission).

in young children^[15], or is impaired by comorbidity or medical therapy, such as in cystic fibrosis^[16], after chemotherapy^[17,18], or following transplantation^[19,20]. In the pediatric population, MBL exerts greatest influence during an immunological “window of vulnerability”, between the decline in maternal passive immunity but before the development of a fully mature adaptive immune system. There is a strong association between MBL deficiency and childhood infection, which has been found for both milder respiratory tract infections managed within the community^[21], as well as more severe infections requiring hospitalization^[15]. In cystic fibrosis (CF), innate immunity is compromised in part by impaired mucociliary clearance and bronchiectasis. In one series of CF patients, those with mutant *MBL2* alleles had worse pulmonary function and shorter survival to end-stage CF^[16]. The same investigators reported successful MBL replacement in the management of one patient with rapidly progressive CF^[22]. Several studies have shown an association between MBL deficiency and risk or severity of infection following chemotherapy^[17,18].

A number of autoimmune disorders are associated with MBL. This may in part relate to the role of MBL in removing pathogens and apoptotic bodies, thus minimizing the emergence of cross-reactivity or auto-immunogenic epitopes^[23]. Inherited deficiencies within the classical complement pathway predispose to systemic lupus erythematosus (SLE), thus it was logical to evaluate the role of MBL

Table 1 Some clinically relevant microorganisms recognized by MBL

Bacteria	Viruses	Fungi	Protozoa
<i>Staphylococcus aureus</i>	HIV-1 and 2	<i>Aspergillus fumigatus</i>	<i>Plasmodium falciparum</i>
<i>Streptococcus pneumoniae</i>	Herpes simplex 2	<i>Candida albicans</i>	<i>Cryptosporidium parvum</i>
<i>Streptococcus pyogenes</i>	Influenza A	<i>Cryptococcus neoformans</i>	<i>Trypanosoma cruzi</i>
<i>Enterococcus spp.</i>	Hepatitis B virus	<i>Saccharomyces cerevisiae</i>	
<i>Listeria monocytogenes</i>	Hepatitis C virus		
<i>Haemophilus influenzae</i>			
<i>Neisseria meningitidis</i>			
<i>Neisseria gonorrhoeae</i>			
<i>Escherichia coli</i>			
<i>Klebsiella spp.</i>			
<i>Pseudomonas aeruginosa</i>			
<i>Salmonella montevideo</i>			
<i>Salmonella typhimurium</i>			
<i>H pylori</i>			
<i>Chlamydia trachomatis</i>			
<i>Chlamydia pneumonia</i>			
<i>Propionibacterium acnes</i>			
<i>Mycobacterium avium</i>			
<i>Mycobacterium tuberculosis</i>			
<i>Mycobacterium leprae</i>			
<i>Leishmania chagasi</i>			

in this condition. A recent meta-analysis concluded that deficient *MBL2* genotypes increase the risk of developing SLE^[24]. Other studies have shown that MBL deficiency increases the risk of SLE-related complications, such as arterial thrombosis^[25]. The effect of variant MBL and risk of vascular complications extend beyond patients with SLE. Several studies have now demonstrated an association between *MBL2* mutations and risk of coronary artery disease^[26-28]. These results have been supported by a population-based study from Denmark, that genotyped 9 245 individuals for *MBL2* coding mutations^[29]. Although MBL deficiency did not greatly increase the rate of morbidity or mortality within the population, those with biallelic mutations had a significantly greater risk of hospitalization for cardiovascular disease compared to those without deficient alleles [RR = 1.2 (1.0-1.4), *P* = 0.02]^[29].

To this point, all of the disease associations presented have identified the wild-type (*A/A*) *MBL2* gene as advantageous. The global preservation of *MBL2* -deficient haplotypes, however, hints at a selective advantage, at least under certain circumstances, of the deficient state. The concept of heterosis, whereby a heterozygous trait may demonstrate a selective advantage, has many well known examples, such as the $\Delta F508$ mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene and resistance to cholera toxin^[30]. MBL-facilitated opsonization and phagocytosis could theoretically enhance the infectivity of some intracellular pathogens. The dichotomous nature of MBL deficiency is supported by several clinical

studies that show it to be protective against several obligate intracellular organisms, including *Mycobacterium leprae*^[31], *M. tuberculosis*^[32], and *Leishmania chagasi*^[33].

Both plasma-derived as well as recombinant MBL are now available for therapeutic use, albeit that the indications for replacement are still evolving. The results from the first phase I trial conducted in healthy, MBL-deficient individuals, has been published^[34]. This study confirmed that MBL replacement is a technically viable option. Phase II trials are eagerly awaited.

MALADIES OF THE BOWEL AND LIVER

Innate immunity has developed multiple strategies for protecting us against microbiological threats. Pattern-recognition molecules, such as toll-like receptors (TLR), *NOD2/CARD15*, and MBL, are particularly important in the alimentary tract, characterized by its large surface area and intimate relationship to the bowel contents, particularly the extreme microbial burden found within the colonic lumen. In addition to initiating inflammation, the capacity for immune tolerance is critical for normal bowel function. Although it is clear that the liver is the chief contributor to plasma MBL, mucosal MBL production may be relevant in localized immune defence, particularly within the alimentary tract^[35]. The following examples present some of the better developed areas of gastroenterological MBL research, including inflammatory bowel disease, carcinogenesis, gastrointestinal and hepatotropic infection, and chronic liver disease.

MBL and inflammatory bowel disease

Inflammatory bowel disease (IBD) is a pathological spectrum encompassing ulcerative colitis (UC), Crohn's disease (CD), and indeterminate colitis. The resultant IBD phenotype is the consequence of multiple interactions between environmental factors, particularly enteric flora, and the host response to this environment, determined by immunogenetic, epithelial, and other non-immune genetic factors^[36]. MBL, as an important component of innate immunity, has engendered considerable research interest. In an early study of 340 unrelated patients with IBD genotyped for *MBL2* exon 1 coding mutations, the frequency of deficient alleles was significantly lower in patients with UC than either the control group ($P = 0.02$), or those with CD ($P = 0.01$)^[37]. This study suggests that MBL deficiency could be protective against UC; alternatively, it could be interpreted that MBL deficiency, in individuals otherwise predisposed to IBD, may skew the phenotype away from the UC spectrum of disease towards CD. This concept is supported by another study that genotyped *MBL2* in patients with CD, UC, or healthy controls^[38]. In this study the allele frequency of coding mutations was approximately 30% in patients with CD, 8% in UC, and 16% in healthy controls. In addition, the frequency of homozygosity or compound heterozygosity for coding mutations (i.e. *O/O* *MBL2* genotype) within the IBD group was significantly higher than in the healthy control population and the association was strengthened if the small number of UC patients were excluded from the analysis (16% *vs* 0%; $P =$

0.05)^[38]. The study also assessed anti-*Saccharomyces cerevisiae* antibody (ASCA) and MBL levels within the same subsets of patients, albeit slightly different numbers within each group^[38]. CD patients with MBL deficiency were significantly more likely to be positive for ASCA and for their lymphocytes to proliferate in response to mannan. Thus, it appears that MBL deficiency could impair normal processing of mannan-expressing microbial antigens, such as those found on the cell surface of many common microorganisms. The accumulated antigens could then stimulate the immune system, and contribute to the production of ASCA and possibly the pathogenesis of Crohn's disease^[38]. ASCA is a well established phenotypic marker of IBD, tending to aggregate with Crohn's rather than the ulcerative colitis phenotype, and within CD the presence of ASCA is particularly associated with the fibrostenosing phenotype and ileal inflammation^[39]. Thus, MBL deficiency might act primarily to influence IBD-specific phenotype in these patients. It should be noted, however, that a follow-up study, testing a larger cohort of CD patients ($n = 241$), failed to confirm the significant association between variant MBL genotypes and ASCA positivity^[40]. The observed trend, however, did show that the frequency of ASCA positivity was proportional to the relative deficiency of the coding genotype, with 54% ASCA positivity for *A/A*, 58% for *A/O*, and 67% for *O/O*^[40]. Nevertheless, further studies into the role of MBL as a marker or regulator of IBD phenotype are warranted. Finally, the contribution of *M. avium* subspecies *paratuberculosis* to the pathogenesis of Crohn's disease is controversial^[41]. Nevertheless, it would be interesting to investigate whether, in a fashion analogous to *M. tuberculosis*, MBL might predispose to Crohn's disease by facilitating the infectivity of this obligate intracellular pathogen.

MBL and coeliac disease

Coeliac disease is an important autoimmune disorder involving the alimentary tract^[42]. The majority of patients with coeliac disease express the major histocompatibility complex (MHC) molecule DQ2 and the remainder usually carry DQ8^[42]. But HLA genes convey only about 40% of the genetic risk, and although 30%–40% of Caucasians carry DQ2 or DQ8, less than 3% of these will develop coeliac disease^[42]. In one study, 117 patients with histologically and serologically confirmed coeliac disease were genotyped for *MBL2* exon 1 mutations, and compared to a healthy blood donor population. There was a significant difference in the frequency of the *O/O* genotype between those with coeliac disease (13%) and the control group (5%) ($P = 0.04$)^[43]. A follow-up study included a detailed assessment of the coeliac disease patients' MHC^[23]. HLA susceptibility alleles and *MBL2* exon 1 coding mutations were genotyped in 147 healthy controls and 149 patients with coeliac disease, enriched with 29 coeliac disease patients known to be negative for DQ2 and DQ8, which is extremely rare^[23]. As in their first study, patients with coeliac disease had a greater frequency of the *O/O* genotype than healthy controls, but in addition, the association between coeliac disease and MBL deficiency was even stronger in the small number of patients negative for DQ2 and DQ8.

It is likely that in those rare cases of coeliac disease that are negative for DQ2 and DQ8, the non-HLA susceptibility genotypes would exert a greater effect. Their study also analyzed apoptosis within small intestinal biopsy specimens, and showed that MBL tended to aggregate to areas of apoptosis within the epithelium. MBL has been implicated in the normal clearance of apoptotic bodies^[44,45]. The authors postulated that the association between MBL and coeliac disease, and indeed other autoimmune conditions, could relate to impaired apoptosis, whereby MBL deficiency impairs the normal removal and clearance of apoptotic cells, that may subsequently reveal previously hidden self-antigen, causing loss of self-tolerance, and spreading of autoimmunity^[23]. The association between variant *MBL2* alleles and coeliac disease has also been confirmed within the Finnish population^[46].

MBL and colorectal cancer

Experimentally there is the suggestion that MBL (both wild-type and the mutant *B* allele) may possess anti-colorectal cancer tumour activity^[47]. *In vitro* MBL binds specifically to oligosaccharide moieties on colorectal cancer cell line SW1116^[47]. The investigators transplanted SW1116 cells subcutaneously in nude mice, resulting in palpable tumor masses at three weeks. In order to evaluate the *in vivo* anti-tumoral activity of MBL, the mice were administered one of four different intra-tumoral injections. The first group received an injection with vaccinia virus carrying the wild-type (*A*) *MBL2* allele, the second group with the variant '*B*' allele and the two control groups received vaccinia virus alone, or saline alone. Intra-tumoral administration of the recombinant vaccinia virus carrying a *MBL2* gene (either the '*A*' or '*B*' allele) significantly reduced tumor size as compared with the two control groups ($P < 0.005$), and also prolonged survival^[47]. These laboratory results have not, however, been reflected in clinical trials. In fact, patients with colorectal cancer have increased activation of the lectin-complement pathway and increased levels of serum MBL^[48]. In patients undergoing surgery for colorectal cancer, however, low preoperative levels of serum MBL has been linked to an increased risk of developing post-colectomy pneumonia^[49]. Most recently, increased preoperative serum levels of MASP2 predicted adverse outcome following colorectal cancer surgery, both in terms of disease recurrence ($P = 0.03$; HR = 1.4, 1.0-2.0) and survival ($P = 0.0005$; HR = 1.4, 1.2-1.7)^[50]. There are several possible explanations for these results. A preoperative elevation in acute phase markers, such as CRP, is known to predict worse outcome^[51], and the elevation in MASP2 may simply reflect a heightened inflammatory state. Alternatively, MASP2 may meaningfully influence tumor progression. Further studies are required to clarify the role of the lectin-complement pathway in cancer.

MBL and gastrointestinal infection

Despite the well-established role of MBL in innate immunity, there have been relatively few studies detailing the clinical effect of MBL deficiency in enteric infections. One notable exception analyzed the association between MBL deficiency and risk of *Cryptosporidium parvum* enteri-

tis. This study included 72 African patients with acquired immunodeficiency syndrome (AIDS) and diarrhea. They were genotyped for exon 1 *MBL2* mutations and had their duodenal aspirates tested for MBL. Patients with biallelic coding mutations (*O/O*) had a significantly greater chance of cryptosporidiosis compared to those who were either wild-type or heterozygous for *MBL2* mutation (*A/A* or *A/O*, respectively) (OR = 8.2; 95% CI: 1.5-42; $P = 0.02$)^[52]. This study places MBL's anti-microbial function back in the context of the 'window of vulnerability' hypothesis. Of further interest from this study was the detection of MBL within some of the duodenal aspirates. The presence of albumin in the intestinal lumen led the authors to postulate that MBL entered the bowel through mucosal leakage of serum; however, local intestinal production could not be excluded. The association between MBL deficiency and cryptosporidiosis was recently confirmed in a second case-control study, this time in young (< 3 year) Haitian children. Mean serum MBL levels were significantly lower in the cases (1110 vs 2395 ng/mL, $P = 0.002$), and 37% of the cases compared to only 10% of the healthy controls were found to be deficient in MBL (level ≤ 70 ng/mL) ($P = 0.005$)^[53]. Unlike the earlier study, *MBL2* genotyping was not performed, and thus MBL deficiency secondary to enteric protein loss, as a consequence of cryptosporidiosis, could not be excluded. When considered together, however, these two studies present compelling evidence for the role of MBL in the host defense against *Cryptosporidium spp.* infection.

Another study analyzed serum MBL levels in a pediatric population presenting with *Escherichia coli* 0157: H7 colitis. MBL levels were measured in patients with uncomplicated *E. coli* 0157:H7 colitis, patients in whom the colitis was complicated by haemolytic uraemic syndrome (HUS), and in normal and disease (rotavirus enteritis) control groups^[54]. MBL deficiency was not associated with an increased risk of either infection nor the complication of HUS, albeit that without analysis of *MBL2* genotype, overall MBL status may be more difficult to assess.

H. pylori is one of the most common human bacterial infections, affecting approximately 50% of humans, although only 10%-20% of those affected will develop a clinical disorder^[55]. Several immunogenetic polymorphisms are associated with clinical outcomes in *H. pylori* infection^[56], as well as with the risk of infection itself^[57,58]. *H. pylori* activates MBL *in vitro*^[59], and a recent study demonstrated that *H. pylori*-related chronic gastritis causes an increase in gastric mucosal MBL expression, but no association was found between *MBL2* genotype and risk of chronic gastritis^[60]. A recent study was performed to investigate whether MBL deficiency increased the risk of *H. pylori* infection^[61]. Two normal populations (166 blood donors and 108 stem cell donors) were included in the analysis. All individuals were genotyped for *MBL2*, had their peripheral MBL activity characterized by level and functional assays, and were tested for serological evidence of *H. pylori* infection. In this study, MBL deficiency did not increase the risk of *H. pylori* infection, and in one population greater MBL activity actually increased the risk of infection.

It is worth noting that MBL has been implicated in

mediating gastrointestinal ischemia/reperfusion injury in mice^[62]. MBL-null mice (deficient in the two murine genes encoding MBL) developed only minor gut injury after induced ischemia/reperfusion insult compared to the wild-type mice. MBL has been implicated as a mediator of ischemia/reperfusion injury in both the myocardium^[63] and the kidney^[64] and thus clinical correlation of MBL status and risk or outcome following mesenteric ischemia may yield interesting results.

MBL and viral hepatitis

MBL was first isolated from hepatocytes^[65], and the liver produces most if not all of the circulating MBL^[20]. There is obviously considerable functional reserve in hepatic MBL production, because in the setting of cirrhosis, unlike many other hepatic proteins, MBL production appears to be increased^[66]. The viral hepatitis have stimulated considerable MBL-related research. In one study of chronic hepatitis B virus (HBV) infection, MBL codon 54 (B) mutations were significantly associated with risk of developing both symptomatic cirrhosis and spontaneous bacterial peritonitis (SBP)^[67]. The increased risk of SBP in patients with MBL deficiency is biologically plausible, given that low levels of ascitic fluid opsonins are important in the pathogenesis of SBP, and MBL deficiency would be likely to compound this deficit^[68]. A second study by the same investigators recently confirmed the association, extending the results from their previous study, to include the low expression haplotype XA as a risk factor, as well as the 'B' allele. The odds ratio for developing cirrhosis and hepatocellular carcinoma was 1.97 for patients with XA and 1.90 for those with YB ($P = 0.002$)^[69]. A study from the U.S. confirmed these findings, which is important given that the age and route of acquisition of HBV may vary between different countries^[70]. It is likely that MBL plays an important role in the pathogenesis of HBV-related chronic disease, even though some small studies have failed to confirm the association^[71,72]. It will be interesting to examine the influence of MBL status upon the rate or type of drug resistance that emerges in individuals during long-term antiviral therapy.

Many of the studies analyzing MBL in chronic hepatitis C virus (HCV) infection have investigated the role of MBL mutations on rate of sustained viral response following interferon alpha (IFN- α) monotherapy. Two studies, from the same Japanese group, reported that patients who failed to eradicate HCV following IFN monotherapy were more likely to have variant *MBL2* alleles, either the 'B' coding mutation^[73,74] or the 'LXP4' haplotype^[73]. A third Japanese study addressed whether MBL deficiency altered the course of HCV chronic liver disease^[75]. In their cross-sectional study, 52 patients with chronic HCV and 50 controls were genotyped for the 'B' coding mutation in exon 1 of *MBL2*. All patients with HCV had the stage and activity of their liver disease categorized as "chronic inactive hepatitis", "chronic active hepatitis" (CAH), or cirrhosis. No significant differences in the frequency of mutations was found between the patients and the controls, but within the HCV-infected group, all of the patients with heterozygous or homozygous codon 54 mutations had either CAH

or cirrhosis, whilst none of those in the "chronic inactive hepatitis" group had mutations. This represented a significantly higher frequency of mutation in the advanced (CAH plus cirrhosis) liver disease group ($P = 0.0405$)^[75]. A final study from Scotland sought to test the Japanese findings in a Caucasoid population^[76]. This study failed to find an association between MBL deficiency and either progression of liver disease or response to IFN- α therapy. This study, however, did not perform *MBL2* genotyping, but stratified MBL concentrations into four groupings for comparison. On balance, there appears to be an association between MBL status and HCV in terms of both disease progression and response to monotherapy, at least in the Japanese population. The development of newer anti-viral treatment regimens, including pegylated-IFN in combination with ribavirin treatment, makes it necessary to re-evaluate immunogenetic influences, at least, any which are hoped to inform therapy.

MBL and liver transplantation

One of the more exciting recent reports regarding MBL and hepatobiliary disease, addressed the role of MBL deficiency following orthotopic liver transplantation (OLT)^[20]. OLT provides the unique opportunity not only to evaluate the role of MBL in post-transplant infection, but also to assess the contribution of hepatic and extra-hepatic MBL production, because in many recipients the *MBL2* genotype will be different in the liver. The study reported the clinical results from 49 transplants, in which 49 of the donors were genotyped for *MBL2*, 25 of the recipients were genotyped, and serum samples were collected from 25 of the recipients to evaluate the change in serum MBL concentration post-transplant. There was an impressive correlation between the risk of post-transplant clinically significant infection and the relative deficiency of the donor *MBL2* genotype, with infection occurring in 12% receiving a wild-type A/A liver, 39% of those receiving an A/O liver, and in 67% of those receiving an O/O donor liver ($P = 0.01$)^[20]. In addition, the post-transplantation serum MBL level was predicted by the hepatic, not the extra-hepatic, genotype. Nevertheless, as only 25 of the recipients, and thus only 25 extra-hepatic genotypes were analyzed, this study was too small to detect more subtle changes in the risk of infection, conferred through local, extra-hepatic MBL production^[77].

MBL and hepatic synthesis

The site of MBL production has been a contentious area. Undoubtedly, the liver produces the majority of MBL and most if not all of the circulating MBL within peripheral blood. This view is supported by the liver transplantation study above, as well as a report documenting that successful allo-SCT failed to correct peripheral blood MBL deficiency^[20,78]. Nevertheless, MBL mRNA is expressed in extra-hepatic tissue^[35,79,80] possibly including haemopoietic lineages^[35], and there have now been two allo-SCT studies that support a contribution from donor (i.e. hematopoietic) *MBL2* genotype, and risk of post-transplant infection^[4,81]. The most important issue is not whether extra-hepatic MBL production significantly influences peripheral

blood MBL levels, but whether it contributes in a clinically meaningful way to local, tissue-specific immunity.

CONCLUSION

MBL has stimulated a great deal of basic and clinical gastroenterological research and has provided new insights to the pathogenesis of infectious and immune disorders within the bowel and liver. The possibility of a local, mucosal effect of MBL as suggested by gene expression^[35,79,80], as well as clinical studies^[23,60] is an exciting discovery. Further work is needed to clarify whether mucosal production occurs, and if so whether it contributes to local immune surveillance in health, or under certain situations, even exacerbates alimentary tract disease.

Despite the promise of replacement therapy and the value of MBL in predicting the risk of disease and disease-specific complications, for now investigation of MBL status remains primarily a research tool. Future studies more rigorously examining MBL status by both measurement of MBL levels and *MBL2* genotyping in large patient cohorts will help clarify the most important disease associations and identify those clinical settings in which MBL replacement therapy is most likely to be beneficial. Now that MBL replacement has been shown to be feasible, the first trials of MBL replacement therapy in several clinical settings, including recurrent infection, severe sepsis, and liver transplantation, are likely to be reported in the next few years. This prospect of MBL replacement therapy represents the culmination of several decades of basic and translational research and is an exciting advance in the field of innate immunity.

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Emerging roles of the intestine in control of cholesterol metabolism

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Abstract

The liver is considered the major "control center" for maintenance of whole body cholesterol homeostasis. This organ is the main site for *de novo* cholesterol synthesis, clears cholesterol-containing chylomicron remnants and low density lipoprotein particles from plasma and is the major contributor to high density lipoprotein (HDL; good cholesterol) formation. The liver has a central position in the classical definition of the reverse cholesterol transport pathway by taking up periphery-derived cholesterol from lipoprotein particles followed by conversion into bile acids or its direct secretion into bile for eventual removal *via* the feces. During the past couple of years, however, an additional important role of the intestine in maintenance of cholesterol homeostasis and regulation of plasma cholesterol levels has become apparent. Firstly, molecular mechanisms of cholesterol absorption have been elucidated and novel pharmacological compounds have been identified that interfere with the process and positively impact plasma cholesterol levels. Secondly, it is now evident that the intestine itself contributes to fecal neutral sterol loss as a cholesterol-secreting organ. Finally, very recent work has unequivocally demonstrated that the intestine contributes significantly to plasma HDL cholesterol levels. Thus, the intestine is a potential target for novel anti-atherosclerotic treatment strategies that, in addition to interference with cholesterol absorption, modulate direct cholesterol excretion and plasma HDL cholesterol levels.

density lipoprotein; Cholesterol absorption; Reverse cholesterol transport

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INTRODUCTION

Maintenance of cholesterol homeostasis in the body requires accurate metabolic cross-talk between processes that govern *de novo* cholesterol synthesis and turnover to adequately cope with (large) fluctuations in dietary cholesterol intake. Imbalance may lead to elevated plasma cholesterol levels and increased risk for cardiovascular diseases (CVD), the main cause of death in Western society. A multitude of epidemiological studies has shown the direct link between high plasma cholesterol, particularly of low density lipoprotein (LDL) cholesterol, and risk for CVD. Treatment of high plasma cholesterol has been focused for many years on interference with cholesterol synthesis by application of statins. Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-controlling enzyme in the cholesterol biosynthesis pathway^[1]. Inhibition of cholesterol synthesis leads to reduced production of very low density lipoprotein (VLDL) particles by the liver and particularly, up-regulation of LDL receptor activity. Both processes contribute to lowering of plasma LDL-cholesterol levels^[2]. Large clinical trials have established the beneficial effects of statin treatment^[3]. However, a relative large number of hypercholesterolaemic patients do not adequately respond to statin therapy or remain at risk for CVD despite substantial reductions in LDL cholesterol^[4,5]. Consequently, alternative strategies are currently actively pursued, particularly high density lipoprotein (HDL)-raising approaches. These approaches are considered particularly promising, as data from epidemiological studies indicate that every 1 mg/dL increase in HDL cholesterol reduces CVD risk by 2%-3%^[6,7]. In addition, strategies aiming at interference with intestinal cholesterol metabolism are gaining interest. A major development has been the introduction of ezetimibe, a potent inhibitor of intestinal cholesterol absorption that reduces plasma LDL-cholesterol by approximately 20% in mildly

hypercholesterolemic patients^[8]. Likewise, phytosterol/sterol (esters)-enriched functional foods have successfully been introduced for lowering of plasma cholesterol levels through interference with cholesterol absorption^[9].

Recently obtained insights in intestinal cholesterol trafficking may open even more promising avenues for further developments. It appears that the intestine actively excretes cholesterol and thereby, significantly contributes to fecal sterol excretion. In addition, it appears that the intestine is an important source of HDL-cholesterol, also known as “good” cholesterol. Thus, the intestine is an attractive target for new therapeutic strategies aimed to alter plasma cholesterol profiles and to reduce the risk for CVD. This review summarizes the important new findings regarding the mechanism(s) of intestinal cholesterol absorption, with specific focus on newly identified transporter proteins, the novel concept of direct intestinal cholesterol secretion and the role of the intestine in HDL biogenesis.

SOME BASIC FEATURES OF CHOLESTEROL

Cholesterol is essential for mammalian life as a structural component of cellular membranes, influencing membrane organization and thereby membrane properties^[10]. Cholesterol is the precursor molecule of steroid hormones and therefore, essential for metabolic control. In the liver, cholesterol can be converted into bile salts, which represents the major pathway for cholesterol metabolism in quantitative sense. Bile salts are amphipathic molecules that facilitate the absorption of dietary cholesterol, fats and fat-soluble vitamins in the small intestine. Recently, it has become clear that bile salts are able to regulate gene expression through activation of the nuclear receptor, the farnesoid X receptor (FXR)^[11-13]. Cholesterol or more correctly, oxidized cholesterol acts as a ligand for the nuclear liver X receptor (LXR or NRH2 or NRH3) and directly contributes to regulation of expression of genes involved in cholesterol, lipid, and glucose metabolism. Accumulation of free cholesterol rather than cholesterylesters, has been shown to induce apoptosis in macrophages by activating the Fas pathway^[14]. Thus, cholesterol is a key component in cellular and whole-body physiology and cholesterol homeostasis is tightly regulated at a variety of levels.

Body cholesterol derives from two sources, i.e., *de novo* biosynthesis and diet. Cholesterol is synthesized from two-carbon acetyl-CoA moieties. The rate-controlling enzyme in the synthetic pathway is HMG-CoA reductase, a highly regulated enzyme that catalyses the conversion of HMG-CoA into mevalonate. Cholesterol itself regulates feed-back inhibition of HMG-CoA reductase activity, as accumulation of sterols in the endoplasmic reticulum (ER) membrane triggers HMG-CoA reductase to bind to Insig proteins, which leads to ubiquitination and degradation of HMG-CoA reductase^[15,16]. In addition, cholesterol regulates the gene expression of HMG-CoA reductase indirectly by blocking the activation of the transcription factor, sterol regulatory element-binding protein 2 (SREBP2). Under low-cholesterol conditions, SREBP2 in the ER binds to the SREBP cleavage activating protein

(SCAP), which escorts SREBP2 to the Golgi. In the Golgi, SREBP2 is cleaved to generate its transcriptionally active form, which activates transcription of the HMG-CoA reductase encoding gene. Upon accumulation of sterols in the ER-membrane, binding of cholesterol to the sterol-sensing domain of SCAP causes a conformation change, which induces binding of SCAP to the ER anchor protein Insig, preventing exit of SCAP-SREBP2 complexes to the Golgi thereby preventing activation of SREBP2^[17].

The contribution of the two sources to the total pool of cholesterol differs between species and prevailing diet composition, but the total cholesterol pool is similar in rodents and humans when expressed on the basis of body weight^[18]. Cholesterol synthesis in the liver is highly sensitive to the amount of (dietary) cholesterol that reaches the liver from the intestine via the chylomicron-remnant pathway^[19]. The Western-type human diet provides approximately 400 mg of cholesterol per day. On top of this, the liver secretes approximately 1 gram of cholesterol into bile per day^[20]. Intestinal cholesterol absorption efficiency in humans is highly variable, ranging from 15% to 85% in healthy subjects^[21]. After uptake by enterocytes, cholesterol is packed with triglycerides into chylomicrons and secreted into the lymph. In the circulation, the triglycerides are rapidly hydrolyzed and free fatty acids are taken up by the peripheral tissues. Cholesterol-enriched chylomicron remnants are subsequently cleared by the liver. Since chylomicron remnants, which contain most of the cholesterol that is being absorbed from the intestine, are rapidly taken up by the liver, interference with the absorption process directly influences hepatic cholesterol synthesis.

The healthy liver is perfectly equipped for handling large amounts of cholesterol. When relatively large amounts of cholesterol reach the liver, *de novo* synthesis and LDL uptake are rapidly down-regulated. In addition, the liver can dispose excess cholesterol molecules in several ways. A rapid response involves esterification of cholesterol by Acyl CoA cholesterol acyltransferase (ACAT) 2 for storage as cholesterylesters in cytoplasmic lipid droplets. Cholesterylester can be hydrolyzed when necessary and this esterification/hydrolysis cycle provides cells with short-term buffering capacity for cholesterol. The liver, like the intestine, is able to produce and secrete VLDL particles, which consist of a neutral lipid core composed of cholesterylesters and triacylglycerols and a monolayer surface containing phospholipids, free cholesterol, and a variety of apolipoproteins. Finally, cholesterol can be converted into bile acids by the hepatocytes, followed by their secretion into the bile along with significant amounts of free cholesterol and phosphatidylcholine. In humans, cholesterol lost via the feces consists of approximately 50% acidic (= bile acids) and 50% neutral sterols, emphasizing the point that conversion into bile acids represents a major pathway for cholesterol elimination.

Peripheral cells, e.g., macrophages, muscle and fat cells, are not able to form lipoproteins or to metabolize cholesterol extensively. Therefore, these cell types depend massively on efflux pathways for removal of their excess cholesterol. It is generally assumed that HDL is the

primary acceptor for cholesterol efflux from cells. HDL cholesterol can subsequently be taken up by the liver for further processing. This pathway is generally referred to as the reverse cholesterol transport (RCT) pathway. The RCT pathway is particularly important for removal of excess cholesterol from macrophages, as accumulation of esterified cholesterol in these cells is considered a primary step in the development of atherosclerosis. Several epidemiological studies have shown that plasma HDL is an independent, negative risk factor for the development of CVD. The common hypothesis is that high HDL cholesterol levels decrease the risk for CVD by removing the excess of cholesterol from the macrophages and enhancing RCT. Recent work, however, indicates that this is an oversimplification and that current concepts of RCT require re-definition^[22]. In addition, the anti-inflammatory and anti-oxidant features of molecules rather than cholesterol associated with the HDL particles, like paraoxonase, platelet activating factor-acetylhydrolase or lysophospholipids, are becoming increasingly apparent^[23-25].

TOWARDS UNDERSTANDING OF INTESTINAL CHOLESTEROL ABSORPTION

In the past years, insight in regulation of cholesterol absorption has greatly increased by identification of transporter proteins involved. In addition, unraveling of molecular regulation of their expression is progressing. Yet, it should be realized that besides transporter proteins, the presence of bile acids in the intestinal lumen is an essential prerequisite for absorption to occur^[26]. Micellar solubilization of (dietary/biliary) cholesterol is necessary for its absorption as exemplified by the fact that fractional cholesterol absorption is virtually zero in bile-diverted rats and *Cyp7a1*-deficient mice with a strongly diminished bile acid pool size^[26].

Identification of novel proteins involved in cholesterol absorption

Cholesterol absorption has long been considered a merely passive process, despite the fact that the process is clearly selective since dietary cholesterol is absorbed with a relative high efficiency whereas structurally similar phytosterols are not. Several candidate intestinal cholesterol transporters have been proposed during the past couple of years, e.g., SR-B1^[27] and aminopeptidase N^[28], but their role (if any) has remained elusive so far. The recent identification of the Niemann-Pick C1 like 1 (NPC1L1) protein as a crucial molecule involved in cholesterol uptake by enterocytes^[29] and of Abcg5 and Abcg8 proteins as (intestinal) cholesterol efflux transporters^[30-32], has provided definite proof that cholesterol absorption is a protein-mediated, selective and active process.

The identification of NPC1L1 is strongly facilitated by the discovery of a powerful cholesterol absorption inhibitor named ezetimibe^[33]. Ezetimibe and analogs comprise a new class of sterol absorption inhibitors that reduce diet-induced hypercholesterolemia in mice, hamsters, rats, rabbits, dogs, monkeys and humans^[8,33-37]. Using a bioinformatics approach, Altmann *et al.*^[29] have

identified the NPC1L1 protein as a putative cholesterol transporter in intestinal cells. NPC1L1 is expressed in the intestine at the brush border membrane and *Npc1l1*-deficient mice show a 69% reduction in fractional cholesterol absorption. Importantly, treatment with ezetimibe could not further reduce fractional cholesterol absorption efficiency in these mice, indicating that NPC1L1 at least is involved in a pathway targeted by ezetimibe^[29]. In support of this, recent studies have shown that ezetimibe glucuronide, the active molecule, indeed binds to cells expressing NPC1L1^[38]. Using intestinal brush border membrane (BBM) fractions, the authors showed that ezetimibe binds specifically to a single site in the brush border membrane and that this binding is lost in BBM fractions of *Npc1l1*-deficient mice^[38]. The exact cellular localization of NPC1L1 is, however, still under debate. Iyer *et al.*^[39] showed that NPC1L1 is glycosylated and enriched in the BBM of rat enterocytes. Davies *et al.*^[40] who were the first to identify NPC1L1 as a homolog of the Niemann Pick type C (NPC) protein^[40], showed in HepG2 cells that NPC1L1 is localized to a subcellular vesicular compartment but not in the plasma membrane. Using immortalized fibroblasts from wild-type and *Npc1l1* knock-out mice these authors also showed that lack of NPC1L1 activity causes dysregulation of caveolin transport and localization, suggesting that the observed sterol transport defect may be an indirect result of the inability of *Npc1l1*-deficient cells to properly target and/or regulate cholesterol transport in the cells.

Another possible mechanism of action of ezetimibe has been proposed by Smart and colleagues^[41]. These authors described the presence of a stable complex of annexin (ANX) 2 and caveolin (CAV) 1 located in enterocytes of zebrafish and mouse. Disruption of this complex by morpholino antisense oligonucleotides in zebrafish prevented normal uptake of cholesterol. Ezetimibe treatment of zebrafish, C57Bl/6 mice fed a Western type diet and LDL receptor knock-out mice disrupted the ANX2-CAV1 complex, suggesting that ANX2 and CAV1 are components of an intestinal sterol transport complex and targets for ezetimibe. Interestingly, C57BL/6 mice fed a standard diet did not show disruption of the ANX2-CAV1 complex upon ezetimibe treatment, but did show decreased cholesterol absorption^[41]. Moreover, recent research using CAV1-deficient mice revealed that inhibition of cholesterol absorption by ezetimibe did not require the presence of CAV1^[42]. In addition, rabbits did not appear to form the ANX2-CAV1 complexes, yet, their cholesterol absorption efficiency was still inhibited by ezetimibe^[43]. Collectively, these studies make a mode of action in which ezetimibe acts by deregulating the ANX2-CAV1 complex less likely.

Other proteins critical in control of sterol absorption are the ATP-binding cassette (ABC) transporter proteins, G5 and G8. ABCG5 and ABCG8 act as functional heterodimers^[44] and are localized at the canalicular membrane of hepatocytes and at the brush border membrane of enterocytes. Mutations in the human genes encoding ABCG5 or ABCG8 have been shown to cause the inherited disease sitosterolemia^[30-32], which is characterized by an accumulation of plant sterols

(e.g., sitosterol, campesterol) in blood and tissues due to their enhanced intestinal absorption and decreased biliary removal. Thus, ABCG5/ABCG8 limit plant sterol absorption by effective efflux back into the intestinal lumen. Since ABCG5/ABCG8 also accommodate cholesterol, as evidenced from the fact that *Abcg5/g8*-deficient mice show a strongly reduced biliary cholesterol secretion^[45], this system also provides a means to control cholesterol absorption efficiency. Yet, *Abcg5* and/or *Abcg8* deficiency in mice clearly enhances phytosterol absorption^[45-47], but reported effects on cholesterol absorption efficiency are minimal^[45,46]. On the other hand, overexpression of *ABCG5* and *ABCG8* in mice as well as pharmacological induction of their expression did lead to a strongly decreased fractional cholesterol absorption^[46,48,49], indicating that ABCG5 and ABCG8 play a role in control of cholesterol absorption under certain conditions.

Other transporter proteins, like the scavenger receptor BI (SR-BI) and ABCA1 have been suggested to play a role in control of cholesterol absorption. In the small intestine, SR-BI is localized both at the apical membrane and at the basolateral membrane of enterocytes, with different expression levels along the length of the small intestine^[50]. It was reported that mice deficient in SR-BI, however, show only a small increase in fractional cholesterol absorption efficiency and a small decrease in fecal neutral sterol output^[51]. On the other hand, intestine-specific overexpression of SR-BI in mice did lead to increased cholesterol and triglyceride absorption in short-term absorption experiments^[52], indicating that SR-BI might have a role in cholesterol absorption.

Although earlier reports^[53] have suggested an apical localization, it is evident that ABCA1 is localized at the basolateral membranes of chicken enterocytes^[54] and human CaCo-2 cells^[55]. The conflicting results yielded in studies assessing intestinal cholesterol absorption in mice lacking *Abca1*^[56,57], suggest that the overall effect of *Abca1* on absorption is very minor. However, as will be described later, this protein does have an important function in intestinal cholesterol metabolism.

After uptake, cholesterol is esterified by the enzyme ACAT 2 in the endoplasmic reticulum (ER) of enterocytes. It was reported that *Acat2*-deficiency in mice on a low-cholesterol chow diet did not affect cholesterol absorption efficiency, however, *Acat2*-deficient mice did show a clear reduction in cholesterol absorption upon feeding a high-fat/high-cholesterol diet and as a consequence, are resistant to diet-induced hypercholesterolemia^[58]. Other proteins crucial for cholesterol absorption are those involved in chylomicron formation, like apolipoprotein B (apoB) and microsomal triglyceride transfer protein (MTP), and proteins involved in intracellular chylomicron trafficking such as SARA2. Mutations in the MTP gene result in abetalipoproteinemia, an inherited human disease characterized by extremely low plasma cholesterol and triglyceride levels and absence of apoB-containing particles. Patients suffer from fat and cholesterol malabsorption and neurological diseases due to malabsorption of lipid-soluble vitamins. Mutations in SARA2 cause chylomicron retention disease or Anderson disease^[59], both of which are characterized by the inability to secrete chylomicrons

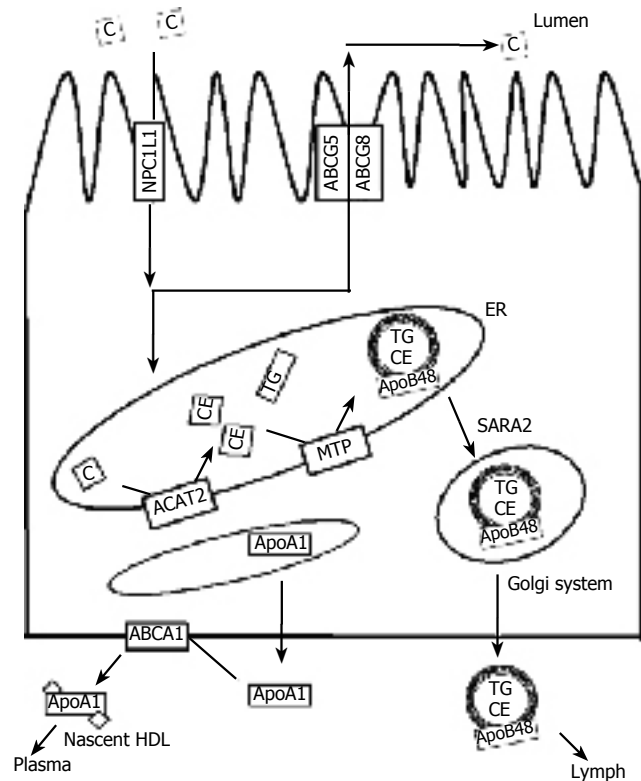


Figure 1 Schematic overview of the major routes of cholesterol in enterocytes. Dietary and biliary cholesterol are taken up via the action of NPC1L1. In the ER, cholesterol is esterified and incorporated into chylomicrons, which are subsequently secreted into lymph. Non-esterified sterols can be re-secreted into the intestinal lumen via the action of ABCG5/G8 or secreted towards ApoA1 via the action of ABCA1. ABCA1, ABCG5, ABCG8: ATP-binding cassette transporter A1, G5, G8; ACAT2: Acyl-coenzyme A: Cholesterol acyl transferase 2; ApoA1, ApoB48, apolipoprotein A1, B48; C: Cholesterol; CE: Cholesteryl ester; ER: Endoplasmic reticulum; MTP: Microsomal triglyceride transfer protein; NPC1L1: Niemann Pick C 1 like 1 protein; SARA2: Sar1-ADP-ribosylation GTPase 2; TG: Triglycerides.

causing severe fat malabsorption and accumulation of chylomicron-like particles in enterocytes. SARA2 belongs to the Sar1-ADP-ribosylation factor family of small GTPases and is involved in intracellular trafficking of chylomicrons through the secretory pathway^[59].

The major routes of cholesterol in enterocytes and the proteins involved are depicted schematically in Figure 1.

Regulation of cholesterol absorption

As indicated above, cholesterol can be taken up from the intestinal lumen by NPC1L1 and effluxed back into the lumen *via* ABCG5 and ABCG8. When both processes are active and present in the same cells, a classical futile cycle arises, enabling very sensitive regulation. Interference with this system has a great potential for reducing plasma cholesterol.

An established application hereof is provided by ezetimibe that interferes with NPC1L1 activity^[29,38]. Lowering of NPC1L1 expression provides another potential means to reduce cholesterol absorption. Mechanisms involved in transcriptional control of NPC1L1 are beginning to be unraveled. The nuclear receptor peroxisome proliferator-activated receptor (PPAR) δ/β (NR1C2) has been shown to decrease cholesterol absorption, presumably by decreasing

NPC1L1 expression^[60]. Activation of PPAR δ/β by the synthetic agonist GW610742 resulted in a 43% reduction of cholesterol absorption in mice, which coincides with unchanged intestinal expression of *Abcg5* and *Abcg8* but a decreased intestinal expression of *Npc1l1*. Treatment of human colon-derived CaCo-2 cells with ligands for PPAR δ/β , but not for PPAR γ or PPAR α , decreased *NPC1L1* expression as well^[60]. Whether PPAR δ/β regulates *NPC1L1* directly or indirectly via transcriptional repression, is still under investigation.

The major regulatory pathways in cholesterol metabolism are controlled by the nuclear receptor liver X receptor (LXR). Two LXR isotypes have been identified in mammals, i.e., LXR α (NR1H3) which is mainly expressed in the liver, kidney, intestine, spleen and adrenals, and LXR β (NR1H2) which is expressed ubiquitously. Natural ligands for both LXRs are oxysterols. After activation, LXR heterodimerizes with retinoid X receptor (RXR)^[61,62]. Activated RXR/LXR heterodimers bind to specific LXR response elements (LXREs) in the promoter regions of their target genes and activate gene transcription. LXR target genes include many genes involved in cellular cholesterol efflux like *ABCA1*, *ABCG1*, *ABCG5*, and *ABCG8*^[53,63,64], genes involved in bile acid synthesis [*cholesterol-7 α -hydroxylase* (*Cyp7a1*)] in rodent models and genes involved in lipogenesis like sterol regulatory element-binding protein (SREBP) 1C, fatty acid synthase (FAS) and *acetyl-CoA* carboxylase (ACC). Global LXR activation by synthetic agonists therefore has a plethora of effects including elevated HDL levels, hypertriglyceridemia, hepatic steatosis, increased biliary cholesterol excretion, reduced intestinal cholesterol absorption efficiency and increased neutral sterol loss *via* the feces^[65,66]. The decreased intestinal cholesterol absorption is primarily due to increased cholesterol efflux of cholesterol towards the intestinal lumen due to increased *Abcg5* and *Abcg8* expression, as fractional cholesterol absorption was reduced upon LXR activation in wild-type mice but remained unaltered in *Abcg5/g8*-deficient mice^[49] and *Abcg5*-deficient mice^[46] under these conditions. Other mechanisms, such as reduced intestinal *Npc1l1* expression after LXR activation contribute to reduced cholesterol absorption, as recently shown in *ApoE2*-knock-out mice^[67].

Dietary phytosterols and phytostanols and their esters have been introduced in functional foods to suppress intestinal cholesterol absorption and hence to reduce the risk for CVD^[9]. Phytosterols and stanols are thought to decrease cholesterol absorption by competing with cholesterol for incorporation into mixed micelles in the intestinal lumen^[68]. However, several recent studies suggested additional mechanisms involving alterations of intestinal gene expression. Igel and colleagues^[69] showed for the first time that phytosterols and stanols are actually taken up by the enterocytes and subsequently re-secreted into the gut lumen, most probably through the action of *Abcg5/Abcg8* transporters. This finding indicated that phytosterols and stanols, in addition to modes of action within the intestinal lumen, exert metabolic actions from inside the enterocytes. Moreover, dietary phytostanol consumption (2.5 g) once a day reduces LDL cholesterol as effective as consumption

of 2.5 g phytosterols ingested in three daily portions^[70], suggesting that luminal concentrations may not be the key to the control of metabolic actions. The identification of a phytosterol-derived agonist for the nuclear receptor LXR^[71] has led to the proposal that phytosterols and stanols decrease cholesterol absorption via activation of intestinal LXR. *In vitro* studies in CaCo-2 cells indicated that phytosterols indeed are able to induce the expression of *ABCA1*, an established LXR target gene^[72]. Recent *in vivo* studies, however, showed that dietary phytosterols and phytostanols decrease cholesterol absorption without activating LXR in rodent models. Field *et al.*^[73] showed that addition of 2% phytosterols to a chow diet do not affect intestinal expression of *ABC sterol transporters* and *Npc1l1* in male golden Syrian hamsters. Likewise, Calpe-Berdien *et al.*^[74] showed very recently that decreased cholesterol absorption upon addition of 2% phytosterol to a Western type diet is not associated with transcriptional changes in *Abca1*, *Abcg5*, *Abcg8* or *Npc1l1* in C57BL/6J, *ApoE*^{-/-} and *LDLr*^{-/-} mice. Plösch and colleagues^[66] have shown similar results using 0.5% phytosterol or phytostanol in a semi-synthetic diet containing 0.2% cholesterol in C57BL mice. Additionally, these authors showed that the plant sterol/stanol-induced reduction of cholesterol absorption in mice is not influenced by *Abcg5*-deficiency (J. Nutr., in press), indicating that intra-luminal events are most relevant for the inhibitory effect of these dietary compounds.

The modes of action of the different cholesterol absorption decreasing compounds are schematically depicted in Figure 2.

NOVEL ROLE OF THE INTESTINE IN REVERSE CHOLESTEROL TRANSPORT

It is clear that the intestine plays a major role in cholesterol homeostasis as a cholesterol absorbing organ. However, recent studies revealed that the intestine also acts as an excretory organ in the reverse cholesterol transport (RCT) pathway^[66,75]. This pathway is classically defined as the HDL-mediated flux of cholesterol from peripheral cells to the liver, followed by its secretion into bile and disposal *via* the feces. RCT is extremely important in prevention of CVD as it removes excess cholesterol from macrophages present in the arterial vessel wall. The amount of cholesterol secreted into bile is substantial. As only part of it is absorbed by the intestine, it contributes significantly to cholesterol loss *via* the feces. However, a novel pathway that contributes to fecal cholesterol loss has recently been established.

Already in the nineteen-sixties, it was suggested that non-dietary cholesterol present in the intestinal lumen consists of a fraction secreted by the liver into the bile and a second fraction directly secreted by the intestine. Measuring dietary cholesterol, cholesterol absorption and cholesterol loss *via* the feces in patients with complete obstruction of common bile duct due to carcinoma of the head of the pancreas unequivocally established the presence of intestinally secreted cholesterol in the feces^[76]. By intestinal perfusion studies in humans, Simmonds *et al.*^[77] have tried to quantify this route. In a triple lumen tube system, perfusion studies were carried out using micellar solutions

with radio-labeled cholesterol. Decrease in specific activity was interpreted as secretion of endogenous cholesterol from the intestine and the contribution of endogenously secreted cholesterol from the intestine was estimated to be about 44% of total fecal output, but direct proof for the existence of this pathway could not be provided^[77].

Since these early experiments, the focus of research has shifted more towards the liver. Biliary cholesterol and bile acid secretions are believed to represent the major pathways for removal of excess cholesterol. However, recent calculations of cholesterol fluxes in different mouse models again emphasize the relevance of intestinal cholesterol secretion. A striking example is provided by the *Cyp7a1*-deficient mouse^[78]. *Cyp7a1* is important for converting cholesterol into bile acids and catalyzing the formation of 7 α -hydroxycholesterol^[79]. As *Cyp7a1* is rate-controlling in this pathway, it is regulated in a complex manner involving multiple nuclear receptors. *Cyp7a1*-deficiency in mice leads to significantly decreased fecal bile acid loss and bile acid pool size. Surprisingly, fecal neutral sterol output is increased two times in *Cyp7a1*^{-/-} mice, although biliary cholesterol concentration remains unaffected^[78]. As dietary intake and cholesterol absorption are known, it can be calculated from these data that direct intestinal cholesterol secretion contributes at least 30% to the increased fecal neutral sterol output.

Plösch and colleagues^[66] showed that the pathway of intestinal cholesterol secretion can be induced in mice by treatment with the synthetic LXR agonist T0901317. In C57BL/6 mice, efflux of cholesterol from the intestinal epithelium into the lumen, calculated from the difference between dietary and biliary input minus fecal output, contributed up to 36% of the total fecal cholesterol loss. Pharmacological LXR activation in these mice tripled the intestinal cholesterol secretion, showing that this represents a valid, inducible pathway for removal of cholesterol in mice.

To further characterize this route, Kruit *et al*^[75] studied the effects of LXR activation by the synthetic agonist GW3965 in wild-type and *Mdr2*-deficient mice. *Mdr2*-Pgp (or Abcb4 according to the new nomenclature) mediates the ATP-dependent translocation of phospholipids at the canalicular membrane of hepatocytes. Consequently, *Mdr2*-deficiency leads to the inability to secrete phospholipids into the bile. Due to the tight coupling of phospholipid and cholesterol secretion, these mice also show a severely impaired biliary cholesterol secretion^[80,81]. Despite the impaired biliary cholesterol secretion, chow-fed *Mdr2*^{-/-} mice showed a similar fecal neutral sterols loss as wild-type mice, suggesting that the intestine indeed contributes to the fecal neutral sterol loss. LXR activation increased fecal neutral sterol output to a similar extent in *Mdr2*^{-/-} and wild-type mice, although biliary cholesterol secretion remained impaired in *Mdr2*^{-/-} mice but increased in wild-type mice. These data show that the increased fecal cholesterol loss upon LXR activation is independent of biliary cholesterol secretion. Although fractional cholesterol absorption decreased to a greater extent in *Mdr2*^{-/-} mice compared to wild-type mice upon LXR activation, it could be calculated that at least 57% of fecal cholesterol originates from intestinal secretion in *Mdr2*^{-/-} mice^[75].

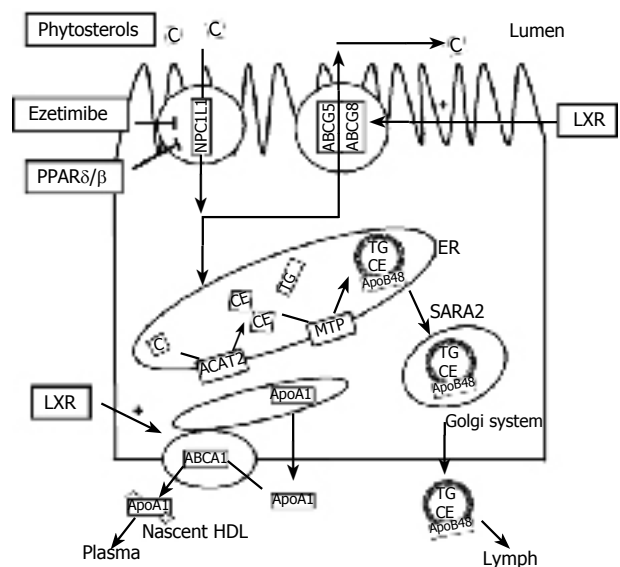


Figure 2 Schematic overview of the regulation of cholesterol transport in enterocytes. Plant sterols, ezetimibe PPAR δ/β agonists and LXR agonists all reduce cholesterol absorption through different mechanisms. Plant sterols interfere with micellisation of cholesterol. Ezetimibe binds to NPC1L1 and thereby interferes with the cholesterol uptake. Agonists for PPAR δ/β reduce expression of NPC1L1 and thereby the amount of NPC1L1 protein. Agonists for LXR increase the expression of ABCG5 and ABCG8 and thereby enhance the efflux of cholesterol towards the intestinal lumen. LXR: Liver X Receptor; PPAR δ/β : Peroxisome proliferators-activated receptor δ/β .

The most intriguing question, namely the origin of intestine-derived cholesterol has remained unanswered so far. Part of the cholesterol could, in theory, originate from enhanced sloughing of intestinal cells or reflect a consequence of increased intestinal *de novo* cholesterol synthesis. Indeed, increased intestinal cholesterol synthesis has been found in *Cyp7a1*^{-/-} mice^[78]. Upon LXR activation, however, intestinal *HMGCoA reductase* gene expression remained unchanged^[66,75], indicative for unchanged cholesterol synthesis, while fecal sterol loss increased 3 times. Staining for the proliferation marker Ki-67 has revealed no signs of increased intestinal cell proliferation upon LXR activation, making the possibility of enhanced cell shedding less likely^[75]. Using intravenously injected radiolabeled cholesterol as a marker, Kruit and colleagues^[75] additionally showed that fecal loss of plasma-derived cholesterol is 1.7-fold higher upon LXR activation in *Mdr2*^{-/-} mice, suggesting that the intestine plays an important role independently of biliary cholesterol in cholesterol transport from plasma to the feces.

Further research should be done to identify the putative proteins involved in this pathway. The sterol efflux proteins, ABCG5/ABCG8, seem to be good candidates, as increased fecal neutral sterol output upon LXR activation requires the presence of Abcg5 and Abcg8^[49] and transgenic mice overexpressing human ABCG5 and ABCG8 (*bG5G8Tg*) showed significantly-increased fecal neutral sterol loss^[48]. However, deficiency of *Abcg5* and/or *Abcg8* leads to only mild^[45,49] or no^[46] decrease in fecal neutral sterol loss and the increased fecal neutral sterol excretion loss in the *bG5G8Tg* mice was inhibited in *bG5G8Tg* mice lacking *Mdr2* (*Mdr2*^{-/-}*bG5G8Tg* mice),

suggesting that biliary cholesterol secretion is responsible for the increased fecal sterol loss in *bG5G8Tg* mice^[82]. However, *bG5G8Tg* mice showed a high expression of human *ABCG5* and *ABCG8* in the liver but their expression in the intestine was far less pronounced^[48]. Thus, the question whether intestinal *ABCG5* and *ABCG8* are important for intestinal cholesterol efflux under normal conditions still remains unanswered.

Virtually nothing is known about transporter systems involved in uptake of plasma cholesterol by enterocytes prior to its excretion into the intestinal lumen. LXR activation can upregulate a number of cholesterol transporters, of which only SR-B1 is known to be involved in cholesterol uptake, at least in the liver. Chow-fed *SR-B1*^{-/-} mice show only a small decrease in fecal neutral sterol loss, suggesting a relatively small contribution of intestinal SR-B1 to the control of fecal cholesterol excretion. However, basolaterally localized SR-B1 in enterocytes could theoretically play a role in cholesterol. When free cholesterol in enterocytes decreases due to activation of *ABCG5* and *ABCG8*, uptake of the sterol from the plasma compartment may become energetically favorable.

INTESTINAL CONTRIBUTION TO HDL BIOGENESIS

The intestine along with the liver, has been known for many years to synthesize and secrete apolipoprotein A-I (ApoA-I), the principal apolipoprotein of HDL. Already in 1977, Glickman and Green^[83] have described the synthesis of ApoA-I by the intestine of rats. One year later, Wu and Windmueller^[84] estimated that intestinally synthesized ApoA-I contributes up to 56% of total plasma ApoA-I in rats. A potential role for the intestine in HDL particle assembly was initially suggested from experiments in hepatectomized dogs and studies describing the presence of HDL in mesenteric lymph^[86-89]. More recently, *in vitro* studies using the human colon carcinoma cell line CaCo-2 showed that basolateral efflux of cholesterol occurs in high density ApoB-free, ApoA-I containing lipoproteins^[90,91].

In addition to ApoA-I, ATP-binding cassette (ABC) transporter 1 (ABCA1) is of crucial importance for HDL formation. Three different groups have independently reported mutations of the *ABCA1* gene as the cause of Tangier disease^[92-94]. Tangier disease is characterized by almost complete absence of plasma HDL, abnormal accumulation of cholesteryl esters in reticuloendothelial cells of many tissues and early incidence of atherosclerosis. No abnormalities in the ApoA-I protein^[95] or in protein synthesis have been found. These findings and the subsequent generation of *Abca1*^{-/-} mice which also lack plasma HDL^[57], underscore that ABCA1 is crucial for HDL formation.

ABCA1 performs the rate-controlling step in HDL formation by mediating the efflux of cholesterol and phospholipids to nascent ApoA-I. ABCA1 is widely expressed throughout the body^[96], however not all tissues are important for the regulation of plasma HDL. Bone marrow transplantation studies in which bone marrow of wild-type and *Abca1*^{-/-} mice was transplanted into *Abca1*^{-/-}

or wild-type mice, respectively, revealed that macrophage expression of *Abca1* contributes only minimally to plasma HDL^[97]. Macrophage ABCA1 is, however, important for the development of atherosclerosis because deficiency of *Abca1* in bone marrow-derived cells increased the susceptibility to atherosclerosis in sensitive strains of mice^[98,99]. Conversely, overexpression of *ABCA1* in bone marrow-derived cells inhibited the progression of atherosclerotic lesions in such mice^[100].

As both the liver and intestine synthesize ApoA-I and express significant levels of ABCA1, they are prone to contribute to plasma HDL levels. Indeed, mice overexpressing human *ABCA1* in the liver and macrophages showed increased plasma HDL levels. Since macrophage ABCA1 can only minimally increase plasma HDL^[97], this indicates that plasma HDL is controlled by hepatic ABCA1. A similar conclusion can be drawn from studies employing adenoviral *Abca1* transfer to mouse liver *in vivo*^[101,102]. Basso *et al* showed that treatment of C57BL/6 mice with adenovirus containing *rABCA1-GFP* resulted in a 2-fold increase in plasma HDL levels. Wellington *et al*^[102] treated mice with increasing doses of *ABCA1*-containing adenoviruses, resulting in a dose-dependent increase in hepatic ABCA1 protein expression. HDL cholesterol was increased in mice injected with low doses of *adABCA1*, but surprisingly higher doses did not further raise plasma HDL levels^[102]. Liver-specific *Abca1* knockdown by 50% in mice using siRNA resulted in a 40% decrease of plasma HDL cholesterol levels, indicating that hepatic *Abca1* expression correlates with plasma HDL levels in mice^[103].

The creation of liver-specific *Abca1* knock-out (*Abca1*^{-L/-L}) mice definitively showed that the liver is the major contributor to plasma HDL as liver-specific deficiency of *Abca1* results in a decrease of plasma HDL cholesterol levels by 80%. Further analysis revealed that *in vivo* catabolism of HDL ApoA-I isolated from wild-type mice was 2-fold higher in *Abca1*^{-L/-L} mice due to a 2-fold higher rate of catabolism of ApoA-I in the kidneys^[104]. These data unequivocally demonstrate that hepatic *Abca1* is responsible for the maintenance of the circulating plasma HDL by direct lipidation of lipid-poor ApoA-I containing particles. These data also show that, although the liver is the major organ responsible for HDL levels, additional extra-hepatic sites also contribute to HDL biogenesis.

To address the contribution of intestinal *Abca1* to plasma HDL, intestine-specific *Abca1* knockout (*Abca1*^{-i/-i}) mice were created using the Cre/Lox system with the Cre transgene under the control of the villin promoter^[105]. Brunham *et al* showed that intestinal *Abca1* deficiency resulted in a 30% decrease in plasma HDL cholesterol levels, indicating that intestinal *Abca1* is critically involved in HDL biogenesis. Combined deletion of both hepatic and intestinal *Abca1* resulted in a 90% decrease of plasma HDL, which was similar to the level found in the whole-body *Abca1*^{-/-} mice, proving that the liver and intestine are really the two major sites for HDL biogenesis. Absence of intestinal *Abca1* resulted in decreased transport of dietary cholesterol into plasma HDL, but total intestinal cholesterol absorption was not affected. Surprisingly, lymphatic HDL content was hardly affected in *Abca1*^{-i/-i} mice. In contrast, HDL was virtually

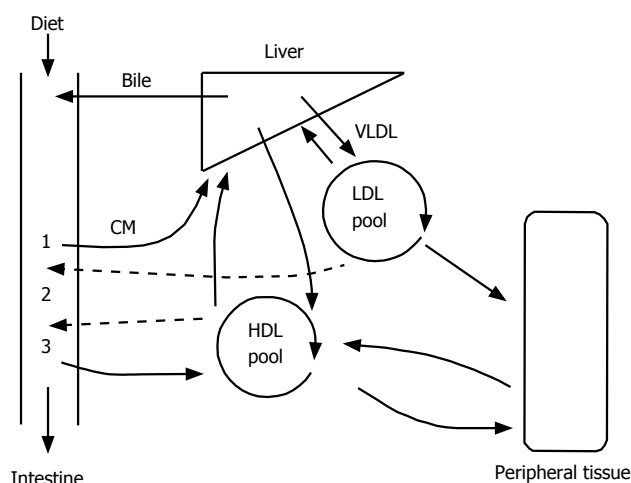


Figure 3 Schematic overview of the involvement of the intestine in cholesterol homeostasis. The intestine is critically involved in the control of plasma cholesterol due to its role in intestinal cholesterol absorption (1), direct cholesterol excretion into the intestinal lumen (2), and HDL biogenesis (3). CM: Chylomicron; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein.

absent in lymph of *Abca1*^{-/-} mice, indicating that lymph HDL originates from the plasma compartment rather than directly from the intestine^[105]. This finding has solved a long-lasting debate on the origin of lymphatic HDL^[83,86-88,106,107]. It would be interesting to see whether lack of intestinal *Abca1* influences the development of atherosclerosis.

Modulation of plasma HDL by intestine-specific LXR activation

As discussed above, LXR is a major regulator of cholesterol metabolism and LXR agonists are considered promising candidates for novel treatment strategies against atherosclerosis. Indeed, treatment of *ApoE*^{-/-} and *LDLR*^{-/-} mice, both are sensitive to atherosclerosis development, with synthetic LXR agonists inhibited the development of atherosclerosis^[108,109]. However, general LXR activation also leads to increased lipogenesis, hypertriglyceridemia and hepatic steatosis in rodents^[65] and is therefore not recommended for its use in humans. Specific LXR activation in the intestine may be beneficial in this respect, as it can theoretically lead to decreased cholesterol absorption, increased intestinal cholesterol excretion and plasma HDL levels. The preliminary data from our laboratory, using an intestine-specific LXR agonist in Wistar rats, showed that intestine-specific LXR activation indeed has the desired effect in this model without adverse effects on triglyceride metabolism.

CONCLUSION

During the past 5 years, a number of developments have greatly contributed to appreciation of the important role of the intestine in maintenance of cholesterol homeostasis (Figure 3). The most important developments include the identification of transporter proteins involved in uptake and secretion of cholesterol by enterocytes, the establishment of the direct cholesterol excretion pathway

of the intestine, and the definition of the role of the intestine in HDL biogenesis.

A wealth of data indicate that the intestine should be considered a promising target for development of anti-atherosclerotic drugs that, in addition to interference with cholesterol absorption, may directly modulate cholesterol excretion and plasma HDL cholesterol levels.

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REVIEW

Trichinosis: Epidemiology in Thailand

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Abstract

Trichinosis is one of the most common food-borne parasitic zoonoses in Thailand and many outbreaks are reported each year. This paper reviews the history, species, and epidemiology of the disease and food habits of the people with an emphasis on the north, northeast, central and south regions of Thailand. The earliest record of trichinosis in Thailand was in 1962 in the Mae Sariang District, Mae Hong Son Province. Since then, about 130 outbreaks have been reported involving 7392 patients and 97 deaths (1962-2005). The highest number of cases, 557, was recorded in 1983. The annual epidemiological surveillance reports of the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand, show that trichinosis cases increased from 61 in 1997 to 351 in 1998. In contrast to these figures, the number of reported cases decreased to 16 in 1999 and 128 cases in 2000. There was no record of trichinosis in 2001, but then the figures for 2002, 2003 and 2004 were 289, 126 and 212 respectively. The infected patients were mostly in the 35-44 years age group and the disease occurred more frequently in men than women at a ratio of 1.7-2.0:1. There were 84 reported cases of trichinosis in Chiang Rai, Nan, Chiang Mai, Si Sa ket, Nakhon Phanom, Kalasin, Nakhon Ratchasima, Nakhon Nayok, Nakhon Pathom and Surat Thani, provinces located in different parts of Thailand in 2005. The outbreaks were more common in the northern areas, especially in rural areas where people ate raw or under-cooked pork and/or wild animals. This indicates the need for health education programs to prevent and control trichinosis as soon as possible in the high-risk areas.

INTRODUCTION

Trichinosis is one of the most widespread helminthic zoonoses. Unlike other parasitic infections, it has been a major public health problem and reported in many Asian countries, including China, Japan, Korea and Thailand^[1-7]. Since 1835, controversy has surrounded the discovery and description of *Trichinella*. In that year the organism, *T. spiralis*, was initially observed by a first-year medical student, James Paget, later famous for other medical achievements, but it was named and described by his professor, Richard Owen. Until recently, that species was the only one known^[8]. However, seven distinct species of *Trichinella* are now recognized, *T. spiralis*, *T. pseudospiralis*, *T. native*, *T. nelsoni*, *T. britovi*, *T. papuae* and *T. murrelli*^[9-16]. In Thailand, human trichinosis was first reported in June 1962. The causative agent of most outbreaks of this disease has been identified as *T. spiralis*^[11], but an outbreak in the Ta Sae District, Chumphon Province in 1994 was due to *T. pseudospiralis*^[17]. Trichinosis infections in humans are characterized by an initial phase dominated by gastro-intestinal symptoms (vomiting and diarrhea), followed by a stage lasting about 2 mo, of fever, sub-cutaneous edema, muscle pain, cachexy, myocardiosis and weakness. Death occurs in up to 40% of cases either due to anaphylactic shock or the consequences of the myocardiosis^[17,18-23]. In eight cases of childhood trichinosis reported in Thailand, major symptoms and signs were fever, myalgia, puffy face and eyelids. Laboratory examinations showed leukocytosis, eosinophilia and elevation of muscle enzymes^[24]. The outbreaks of trichinosis in Thailand seem to depend in part on the density of *Trichinella* contamination in domestic and wild animals, but they are also influenced by social and other factors, including people's eating habits^[25-30]. This study includes a review of the history, species, epidemiology of trichinosis and food habits of Thai people based on previous studies and the annual epidemiological surveillance reports from 2002-2005 from the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand.

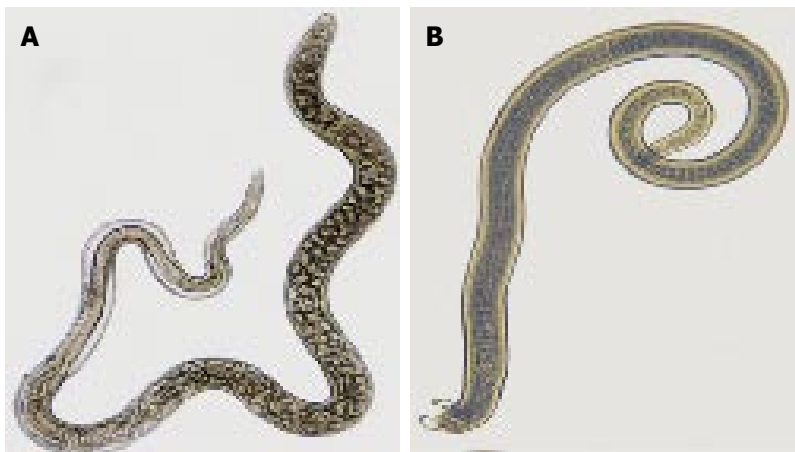


Figure 1 *T. spiralis* in Thailand^[50]. **A:** Adult female *T. spiralis* with fully formed larvae in uterus; **B:** Adult male *T. spiralis* claspers on tail (lower end).



Figure 2 *T. pseudospiralis* in Thailand^[51]. **A:** Adult male *T. pseudospiralis*; **B:** Adult female *T. pseudospiralis* containing eggs and larvae.

HISTORICAL REVIEW OF TRICHINOSIS IN THAILAND

The first outbreak of trichinosis in Thailand was in 1962 and involved 56 patients resulting in 11 deaths in the Mae Sariang District, Mae Hong Son Province. Meat from pigs was the source of outbreak^[1]. The number of outbreaks has tended to increase in recent years. The annual epidemiological surveillance report dated 15 November 2005 recorded 7392 patients and 97 deaths. The highest annual number of hospital recorded trichinosis cases was 557 in 1983. This figure is considered an underestimation of the actual number of cases involved in the outbreaks^[31-36]. In April 1973, an outbreak of trichinosis occurred in the Mae Sruay District, Chiang Rai Province. Thirty-one persons were involved, ranging from 9 to 72 years, and one adult female died^[37]. In 1980, trichinosis was reported in the Pluak Dang District of Rayong Province, the infection being caused by the consumption of wild squirrel^[38,39]. An epidemic of trichinosis involving 177 patients and 13 deaths occurred in April 1981 in Kok-Ta-Back Village, Nong-Pai District, Petchabun Province, and reported the fourteenth outbreak of human trichinosis in Thailand^[40-42]. Khambooruang^[43] reported 118 discrete outbreaks of the disease involving 5400 patients and 95 deaths. In the south of Thailand, an outbreak of trichinosis affecting 59 individuals resulting in one death occurred in Chumporn Province during 1994-1995. This was the first report of an epidemic of human infection caused by *T. pseudospiralis*^[7].

Takahashi *et al.*^[35] reported 120 outbreaks from 1962 to 2000 involving nearly 6700 patients and 97 deaths. The highest number of cases was in Chiang Mai, Chiang Rai and Nan provinces, 1776, 1739 and 894 respectively. Chotmongkol *et al.*^[7] presented the case of a 49-year-old man with progressive generalized muscle hypertrophy and weakness for 3 mo. Histologic findings from muscle biopsy demonstrated a nurse cell-larva complex. Treatment with albendazole resulted in a very favorable outcome. Trichinosis remains a major public health problem in Thailand, often associated with rural people celebrating local and traditional festivals, such as the northern Thai New Year and wedding ceremonies, at which raw and/or under-cooked wild animals are eaten.

SPECIES OF TRICHINOSIS IN THAILAND

Seven species belonging to the *Trichinella* genus, five with encapsulated larvae and two with non-encapsulated larvae in host muscles and three additional genotypes, have been described to date: *T. spiralis*, *T. nativa*, *T. britovi*, *T. murrelli*, *T. nelsoni*, *T. pseudospiralis*, and *T. papuae*. In Southeast Asia, *T. spiralis* and *T. pseudospiralis* have been documented in domestic animals and/or humans in Cambodia, Indonesia (Bali and Sumatra), Lao PDR, Malaysia, Myanmar and Thailand^[10,12,14,15,44]. In Thailand, the causative agent of most outbreaks of trichinosis has been identified as *T. spiralis*^[1] (Figure 1). Meanwhile, Jongwutiwes *et al.*^[7] reported human infection by *T. pseudospiralis* (Figure 2). An outbreak

of trichinosis affecting 59 individuals, of whom one died, occurred in south Thailand during 1994-1995. After that, there were no reports of other species of *Trichinella* in this country in either humans or animals^[35]. Until recently, *T. spiralis* and *T. pseudospiralis* were the only human-infecting species in Thailand.

EPIDEMIOLOGY OF TRICHINOSIS IN THAILAND

Trichinosis is more common in temperate regions than in tropical regions. The epidemiology of trichinosis was first reported in 1962 in patients who consumed pig meat^[1]. The second outbreak was in 1963 at Prao District, Chiang Mai Province. Since then, outbreaks have occurred each year, mostly in the northern part of Thailand where people have eaten raw or under-cooked pork and/or wild animals^[45-47]. The annual epidemiological surveillance reports indicated that trichinosis cases increased from 61 in 1997 to 351 in 1998. In 1999 and 2000, the number of reported cases decreased to 16 and 128 respectively. No cases were recorded in 2001, hospital based or by the Bureau of Epidemiology, that clearly showed a human trichinosis case this year, but then 289, 126 and 212 occurred in 2002, 2003 and 2004 respectively. In 2005, 75 cases were reported by the Bureau of Epidemiology, Department of Disease Control, and Ministry of Public Health (Figure 3). Since then, about 130 outbreaks have been reported totaling 7392 patients and 97 deaths.

Since 2002, the distribution of human trichinosis cases by age groups has been considered by the annual epidemiological surveillance reports which data were the hospital based. The youngest patient was about 1 year old. Charkrit^[44] reported a patient of the same age. It is not uncommon to see patients in the 10-14 and 65+ age groups, but most patients are in the age 35-44 groups, morbidity rate was 0.04 per 100 000 of people (Figure 4). Infection occurs in men more frequently than women at the ratio of 1.7-2:1 (calculated from trichinosis cases of 2002-2005). This result was similar to the 1.2-2.6:1 found by Charkrit^[44].

The epidemiological surveillance reports of trichinosis have been conducted almost every year and data investigation reveals that the outbreaks have occurred predominantly in rural areas. The north part of Thailand is responsible for 96.4% of all cases reported from 1962 to 2000^[35]. The annual epidemiological surveillance reports from 2002 to 2005 found consistently high numbers of cases (289, 126, 30 and 60 respectively) in the north region. The figures for 2004 reported 124 in the northeast, the first time that a region other than the north has had the highest number of cases. Only small numbers of trichinosis cases were recorded in the central and south regions in 2005 (Figure 5). In 2005, 75 trichinosis cases were reported, the highest number occurring in October, August, March and September, 36, 16, 7 and 5 respectively. The cases were reported in Chiang Rai, Nan, Chiang Mai, Si Sa ket, Nakhon Phanom, Kalasin, Nakhon Ratchasima, Nakhon Nayok and Surat Thani, all provinces located in different parts of Thailand. The main age group was 35-44 years and the

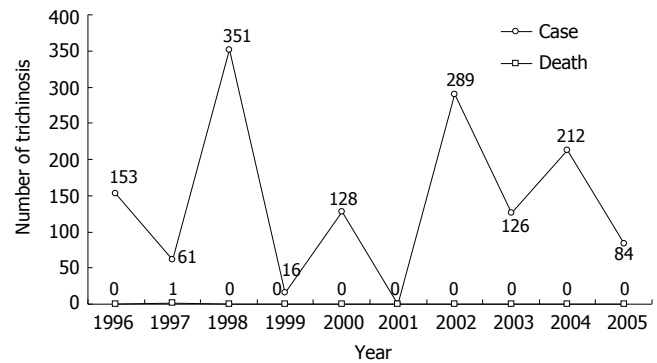


Figure 3 Trichinosis cases in Thailand from 1995-2005.

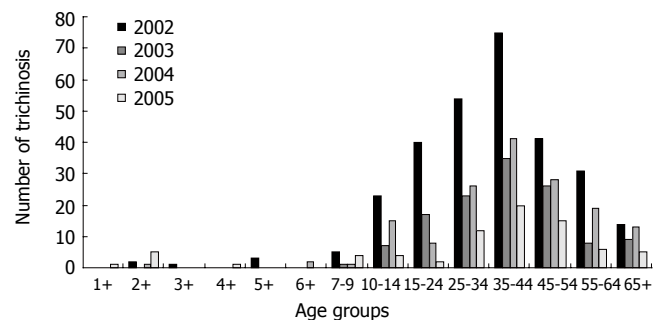


Figure 4 Human trichinosis cases in Thailand from 2002-2005.

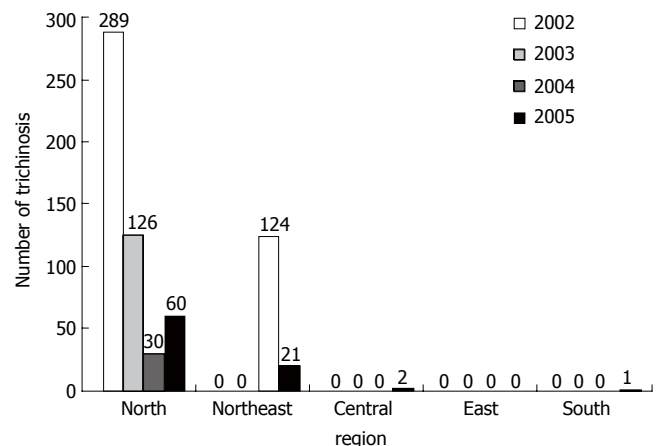


Figure 5 Human trichinosis by region in Thailand from 2002-2005.

youngest patient was 1.

Most outbreaks occurred in the north region, including 60.84% of all cases reported from 1962 to 2005. These results were different from those of Takahashi *et al* (2000) that reported the north region was responsible for 96.4% of all cases. The most severely affected areas in the north region were the highland provinces of Chiang Rai, Nan, Chiang Mai, Mae Hong Son and Payao. (Figure 6). The numbers of cases in other parts of Thailand were very few. In the central region, Uthai Thani, Karnchanaburi Nakhon Pathom and Nakhon Nayok provinces reported 0.28% of the total number of cases. Chumporn, Songkla and Surat Thani were the only three provinces of the south region in

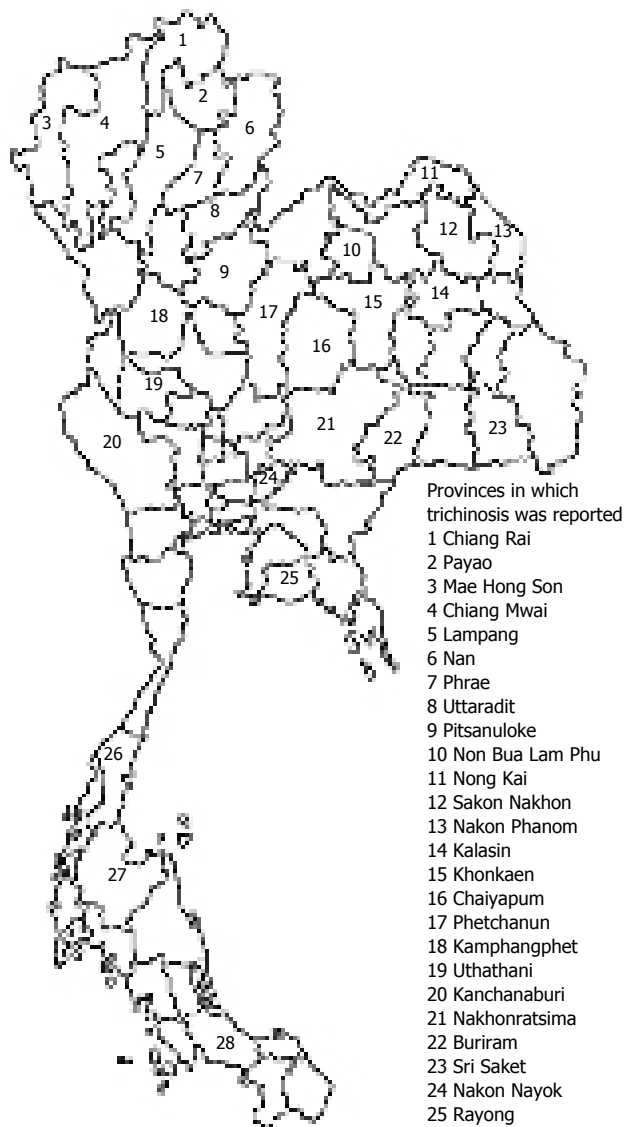


Figure 6 Provinces trichinosis reported in Thailand from 1962-2005.

Table 1 Distribution of trichinosis in humans by province in Thailand from 2002-2005

Region	Province	Total cases (%)
North	Chiang Mai, Chiang Rai, Nan, Phrae, Uttaradit, Payao, Mae Hong Son, Petchabun, Kamphaengphet, Pitsanuloke, Lampang	60.87
Northeast	Nong Bua Lam Phu, Buri Ram, Kalasin, Sakon Nakorn, Sri Saket, Nong Khai, Khon Kaen, Nakhon Phanom, Nakhon Ratchasima	38.59
East	Rayong	0.18
Central	Uthai Thani, Kanchanaburi, Nakhon Nayok, Nakhon Pathom	0.18
South	Chumphon, Songkla, Surat Thani	0.18

which cases of trichinosis were observed, these accounting for only 0.28% of cases (Table 1). The northeast of Thailand was responsible for the highest number of cases in 2004 and the second highest (38.5%) in 2005. Provinces



Figure 7 The dishes of raw or under-cooked wild boar are the favorite local foods that are the major sources of infection in Thailand. A: Lahb is made from wild pig or reptile; B: Nham is also made from wild pig and fermented for a few days.

involved were Nong Bua Lam Phu, Buri Ram, Kalasin, Sakon Nakorn, Sri Saket, Nong Khai, Khon Kaen, Nakhon Phanom and Nakhon Ratchasima. These results showed trichinosis as a serious problem, particularly in the north and northeast regions of Thailand.

SOURCE OF THAI HUMAN INFECTION

Trichinosis is a parasitic disease of mammals caused by the nematode parasite *Trichinella* spp. It has an important zoonosis with humans becoming infected by eating raw or inadequately cooked infected meat. Infection is more common in omnivores (horses, humans, pigs and rats) and carnivores (cats, dogs, and seals). Pigs and rodents seem to play the most important role in the epidemiology of the disease. The main source of infection in Thailand has been pigs, but wild boar, jackal and black bear were also reported as sources of trichinosis^[45,46]. All trichinosis cases gave a history of having consumed raw pork in the form of “lahb” and “nahm,” favorite dishes of north Thailand^[37] (Figure 7). Lahb is made from chopped raw pork mixed with lemon juice, roasted rice powder, finely cut red onion and parsley (Figure 7A). Nham is also made from chopped raw pork mixed with salt, garlic and chili, tightly wrapped in banana leaves for a few days for fermentation (Figure 7B)^[35]. Some Thai dishes are proven as viable *T. spiralis* larvae sources due to cooking procedures^[47]. Srikitjakarn *et al*^[48] reported *T. spiralis* was found in 1.67% of 421 dogs in Tarae District, Sakonnakhon Province. Raw dog meat was a source of infection in Kaeng Khlo District, Chaiyaphum Province

in December 1984^[49]. The incidence of *T. spiralis* larvae in dog meat in the areas favoring dog meat consumption is a major public health problem in the future. The major source of infection is wild boar, free roaming pigs located in the north, and wild animals from Laos and Myanmar sold in Thailand^[2].

In conclusion, various studies of trichinosis in Thailand since the first outbreak up until recent years have shown that most of the outbreaks occurred in the north of the country, an area in which some of the favorite traditional dishes involve meat from pigs and wild boars, often eaten raw or under-cooked. No vaccines have yet been developed. Treatment exists for humans if diagnosis is done promptly. Better prevention and control of trichinosis require health education to stop the consumption of infected and under-cooked meats.

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COLORECTAL CANCER

Identification of specific genes and pathways involved in NSAIDs-induced apoptosis of human colon cancer cells

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cancer cells by NSAIDs may explain in part, their inhibitory action on colon cancer growth.

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Abstract

AIM: To study whether indomethacin (IND), a nonselective cyclooxygenase (COX) inhibitor or NS-398 (NS), a COX-2-selective inhibitor, induces apoptosis in human colon cancer cells and which apoptosis-related genes and pathways are involved.

METHODS: Human colon cancer Caco-2 cells were treated with either: placebo, IND (0.05-0.5 mmol/L) or NS (0.01-0.2 mmol/L) for 1, 5 and 18 h. We then studied: (1) Cell death by the TUNEL method, (2) mRNA expression of 96 apoptosis-related genes using DNA microarray, (3) expression of selected apoptosis related proteins by Western blotting.

RESULTS: Both IND and NS induced apoptosis in 30%-50% of Caco-2 cells in a dose dependent manner. IND (0.1 mmol/L for 1 h) significantly up-regulated pro-apoptotic genes in four families: (1) TNF receptor and ligand, (2) Caspase, (3) Bcl-2 and (4) Caspase recruiting domain. NS treatment up-regulated similar pro-apoptotic genes as IND. In addition, IND also down-regulated anti-apoptotic genes of the IAP family.

CONCLUSION: (1) Both non-selective and COX-2-selective NSAIDs induce apoptosis in colon cancer cells in a dose dependent manner. (2) Both NSAIDs induce apoptosis by activating two main apoptotic pathways: the death receptor pathway (involving TNF-R) and the mitochondrial pathway. (3) IND induces apoptosis by up-regulating pro-apoptotic genes and down-regulating anti-apoptotic genes, while NS only up-regulates pro-apoptotic genes. (4) Induction of apoptosis in colon

INTRODUCTION

Colorectal cancer (CRC) is the second most lethal cancer in the USA. Numerous studies suggest that nonsteroidal anti-inflammatory drugs (NSAIDs) have antineoplastic effects. Epidemiological studies have shown a reduction in the risk of death from colorectal cancer associated with the use of NSAIDs^[1]. Perhaps the most compelling evidence for the chemopreventive role of NSAIDs is provided by the clinical studies in patients with familial adenomatous polyposis (FAP), an inherited predisposition for CRC^[2-6]. In these studies, the NSAID sulindac and the selective cyclooxygenase-2 inhibitor (COX-2) celecoxib, reduced both the number and the size of colorectal adenomas in FAP patients. Similarly, NSAIDs prevent tumor formation in a variety of animal models of CRC^[7,8].

How exactly do NSAIDs exert their effects to prevent tumorigenesis? A large body of evidence suggests that NSAIDs exert their chemopreventive actions by: (1) inhibition of cell growth and proliferation and (2) induction of apoptosis^[9]. The mechanisms by which NSAIDs achieve these processes are still incompletely understood. One widely studied mechanism is *via* the COX-2 dependent pathways.

NSAIDs are known as the inhibitors of the cyclooxygenase (COX-1 and COX-2) enzymes, which are involved in the conversion of arachidonic acid to eicosanoids. COX-1 is expressed constitutively in a variety of tissues and plays a role in platelet aggregation and gastric cytoprotection. COX-2, is inducible particularly during inflammation in cells such as endothelial cells, macrophages and intestinal epithelial cells^[10]. Interestingly,

COX-2 expression has been shown to be dramatically increased in colon cancer cells compared to normal colonic mucosa and is associated with a several-fold increase in concentration of prostaglandin E₂ (PGE₂)^[11-13]. Studies from our lab demonstrated that COX-2 generated PGE₂ promotes colon cancer cell growth by transactivating epidermal growth factor receptor^[14]. The increased expression of COX-2 also seems to prevent cancer cells from undergoing programmed cell death, at the same time, promoting angiogenesis, enhancing metastatic potential and modulating cell proliferation. Direct evidence for the role of COX-2 in colon cancer tumorigenesis has been reported in many studies. Using APC knockout mice, which develop polyps in their intestinal tracts due to the mutation of the APC gene, Oshima and colleagues were able to show a significant reduction of intestinal polyps when either one or both of COX-2 alleles were deleted^[15,16]. Furthermore, the COX-2 selective inhibitor NS-398, has been reported to suppress tumor growth in different cancer cell lines, to induce apoptosis in human colon cancer cells and to have anti-angiogenic effects^[9,17-19].

However, recent data suggest that COX-2 may not be the sole target for the action of NSAIDs. The anti-neoplastic effects of NSAIDs are also seen in tumor cells that do not express COX^[20]. Similarly, NSAID derivatives such as sulindac sulfone, which lacks the ability to inhibit COX, is shown to inhibit colon cancer growth^[21]. Furthermore, NSAIDs affect other mechanisms that regulate cell proliferation and death. For example, NSAIDs regulate PPAR δ , the NF κ B pathway and the lipoxigenase pathway, all of which play a critical role in the regulation of cell survival and death^[22-24].

In order to better understand the complex mechanisms underlying the cancer chemopreventive properties of NSAIDs, it is important to understand the signaling pathways and the genes that directly lead to the inhibition of proliferation and/or the induction of apoptosis in colon cancer cells. With the recent development of DNA microarray technology, identification of genes and pathways can be easily achieved by using microarray chips that contain the genes of interest.

This study was aimed to provide in depth insight into the pro-apoptotic actions of Indomethacin (IND), a nonselective COX inhibitor and NS-398 (NS), a COX-2 selective inhibitor on colon cancer Caco-2 cells, and identify specific pathways and genes involved.

MATERIALS AND METHODS

Cell culture

Human colon cancer (Caco-2) cells (ATCC, Rockville, MD, USA) were grown in DMEM/F12 medium supplied with 20% fetal bovine serum and antibiotics at 37°C in a humidified incubator containing 50 mL/L of CO₂.

Treatment of cells with Indomethacin and NS-398

A 100 mmol/L stock solution IND (Sigma Chemical, St. Louis, MO, USA) was freshly prepared by dissolving the compound in 0.2 mol/L Na₂CO₃ and 1 mol/L NaH₂PO₄, and kept at 37°C before treatment. A 50 mmol/L stock solution of NS (Cayman Chemical, Ann Arbor, MI)

was prepared by dissolving the compound in dimethyl sulfoxide. Caco-2 cells were plated in dishes (100 mm × 20 mm) at a density of 1 × 10⁶ and kept in serum-free medium for 24 h before treatment. The cells were subsequently treated with IND (0.05 mmol/L, 0.1 mmol/L and 0.5 mmol/L) or NS (0.01 mmol/L, 0.1 mmol/L and 0.2 mmol/L) for 1, 5 and 18 h at 37°C.

Cell death detection assay

Cells were plated in 8-well chamber slides and grown until 50%-75% confluent. They were subsequently treated with IND and NS in the same way as described above. Cell death was evaluated by terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) using a commercial kit (Roche Diagnostics, Indianapolis, IN, USA, Cat No. 1684817). Briefly, cell monolayers were air-dried and fixed with 4% paraformaldehyde for 1 h at room temperature. 3% H₂O₂ was then used to block endogenous peroxidase. The TUNEL reaction mixture was applied to the cells and incubated for 1 h. Following incubation with anti-fluorescein antibody conjugated with horseradish peroxidase (HRP), diaminobenzidine substrate was then used as developing the agent. Cells treated with culture medium without NSAIDs served as controls. Apoptosis was evaluated by counting the number of apoptotic and total cells in five random fields per chamber in each slide. Apoptotic index was expressed as percentage of apoptotic cells in total number of counted cells.

cDNA microarray study

Serum starved Caco-2 cells were treated with IND or NS in the same way as described above. Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA, Cat No. 15596-026). Equal amount of RNA from each treatment was used as templates for cDNA synthesis. Radiolabeled probes were synthesized using ³²P dCTP following the manufacturer's protocol provided with the microarray study kit (GEArray Q series, SuperArray Inc. Bethesda, MD, USA). The denatured cDNA probes were added to the arrays containing the genes to be studied. Each of the arrays contains 96 cDNA fragments from genes associated with a specific apoptotic pathway. After overnight hybridization at 60°C with continuous agitation, the arrays were washed for 4 × 15 min with each washing solution (2 × SSC, 1% SDS, then 0.1 × SSC, 0.5% SDS). The arrays were then developed using X-ray films. Quantification of the images was accomplished by the ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). Results were expressed in fold change.

Western blotting

Serum starved Caco-2 cells were treated with IND or NS in the same way as described above. Total protein was isolated in a lysis buffer containing 20 mmol/L Tris-HCl pH 7.5, 1 mmol/L EDTA, 300 mmol/L NaCl, 0.2 mmol/L Sodium vanadate, 1% Triton x-100 and 1% NP-40. Equal amount of protein lysate from each treatment was separated by SDA-PAGE and then transferred electrophoretically to a nitrocellulose membrane. The blots were probed with the following antibodies: APAF1

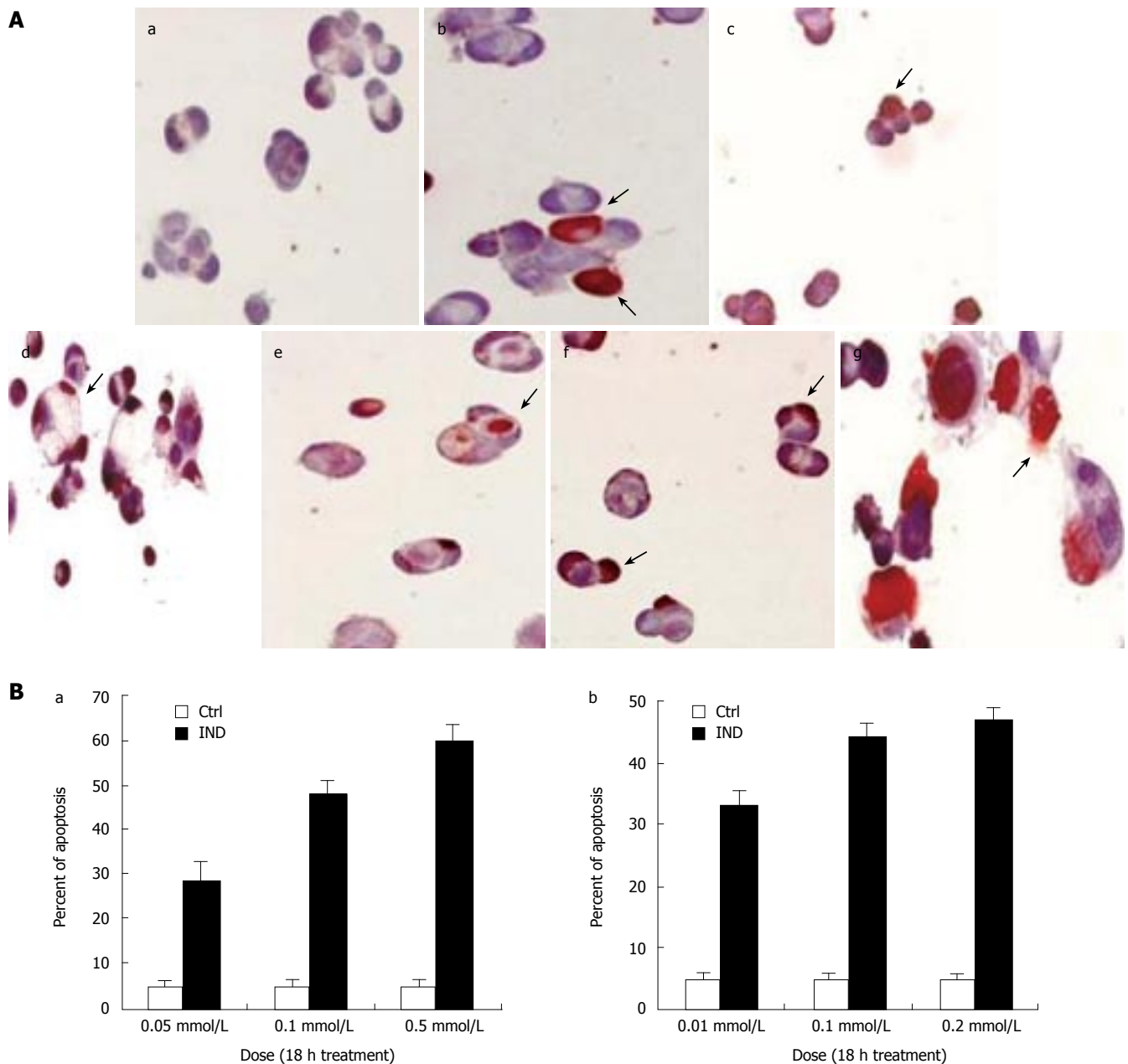


Figure 1 A: TUNEL staining of Caco-2 cells. IND and NS treatment cause apoptosis in a dose dependent manner after 18 h. (a) Control; (b) IND 0.05 mmol/L; (c) IND 0.1 mmol/L; (d) IND 0.5 mmol/L; (e) NS 0.01 mmol/L; (f) NS 0.1 mmol/L; (g) NS 0.2 mmol/L. Experiments performed in triplicates; B: Apoptosis in Caco-2 cells is dose dependent after treatment with (a) IND and (b) NS for 18 h. Experiments performed in triplicates.

(apoptotic protease activating factor), BAK (BCL2-antagonist/killer 1), BAX (BCL2-associated X protein), DFFA (DNA fragment factor-45), XIAP (X-linked IAP) and Survivin. The signal was visualized by the chemiluminescence method using ECL detection reagents (Amersham Life Sciences, Arlington Heights, IL). Signals were quantified by densitometry scanning.

Statistical analysis

Results from the TUNEL study are expressed as the mean \pm the standard error. Student's *t* test was used to determine statistical difference between controls and treatments. A *P* value of < 0.05 was considered statistically significant. Comparisons of data between multiple groups were performed with ANOVA.

RESULTS

Apoptotic cell death detection

Using TUNEL method, we found a significant increase in apoptotic cell death in Caco-2 cells treated with both the nonselective COX inhibitor IND and the COX-2-selective inhibitor NS as compared to placebo. The IND and NS-induced apoptotic cell death was dose dependent (Figure 1A and B). Treatment with IND at 0.05 mmol/L, 0.1 mmol/L and 0.5 mmol/L resulted in $28\% \pm 5\%$, $48\% \pm 3\%$ and $60\% \pm 4\%$ of apoptosis respectively (all $P < 0.001$ *vs* control). On the other hand, treatment with NS at 0.01 mmol/L, 0.1 mmol/L and 0.2 mmol/L induced apoptosis in $33\% \pm 3\%$, $44\% \pm 2\%$ and $47\% \pm 1\%$ respectively (all $P < 0.001$ *vs* control). In addition, at 0.1 mmol/L concentration, IND induced apoptosis in $33\% \pm 4\%$, 31%

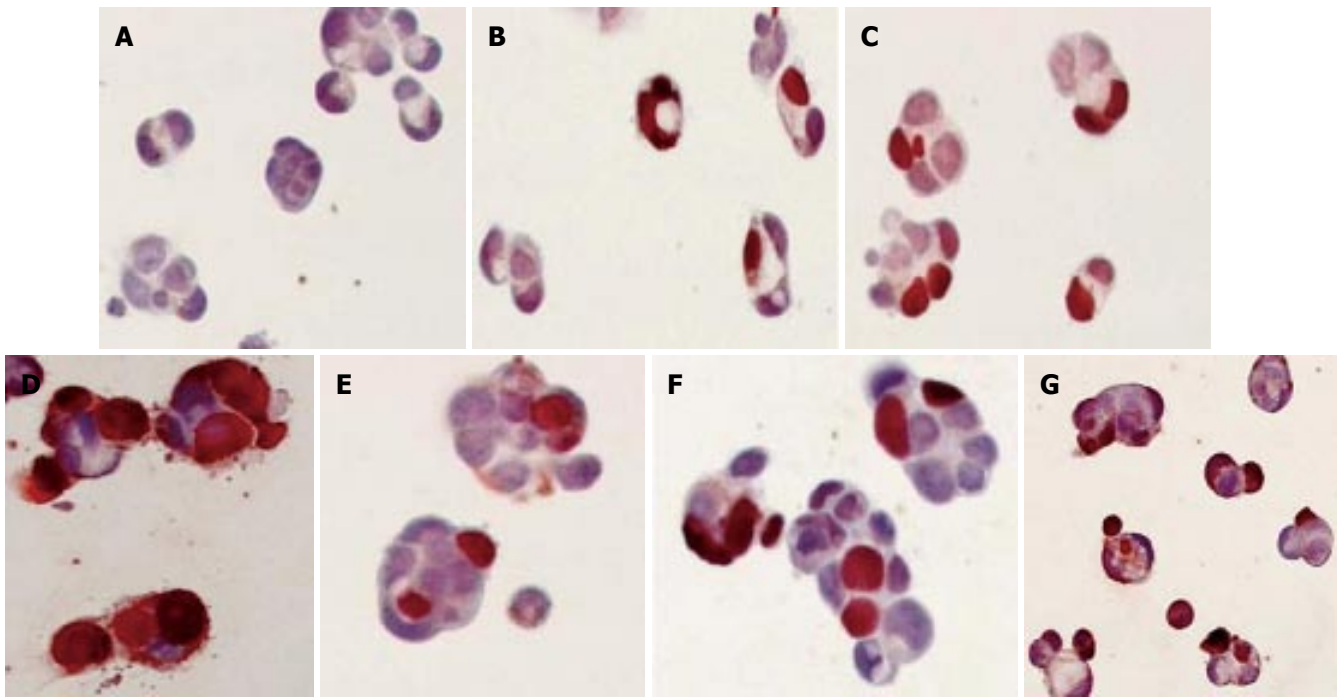


Figure 2 TUNEL staining of Caco-2 cells treated with IND (0.1 mmol/L) and NS (0.1 mmol/L) for 1, 5 and 18 h. (A) Control; (B) IND for 1 h; (C) IND for 5 h; (D) IND for 18 h; (E) NS for 1 h; (F) NS for 5 h; (G) NS for 18 h.

$\pm 2\%$ and $48\% \pm 5\%$ (all $P < 0.001$ *vs* control) of cells and NS induced apoptosis in $29\% \pm 3\%$, $26\% \pm 4\%$ and $46\% \pm 6\%$ (all $P < 0.001$ *vs* control) of cells at 1, 5 and 18 h, respectively (graph not shown).

IND and NS treatment induced morphological changes consistent with apoptosis (cell shrinkage, condensation of chromatin, cytoplasmic budding, apoptotic bodies), which occurred as early as 1 h post-treatment and became extensively evident in 5 and 18 h (Figure 2).

cDNA microarray study of 96 apoptosis related genes

IND treatment (0.1 mmol/L for 1 h) caused a significant up-regulation of pro-apoptotic genes in mainly four families: (I) TNF receptor and ligand (TRAIL-R4-A by 27-fold, TNFSF11 by 3-fold); (II) Caspase (CASP4 by 14-fold, CASP6 by 19-fold); (III) BCL-2 (BCL2L11/bimL by 6-fold, Blk by 10-fold); (IV) Caspase recruiting domain (Apaf-1 by 3-fold, NOD/CARD4 by 14-fold).

In addition, IND significantly up-regulated other genes such as DNA fragmentation factor 45/DFFA (by 48-fold) and CRADD (by 24-fold). In addition to the genes up-regulated by IND, NS up-regulated some other genes within these families such as CASP13 (7-fold), Asc (5-fold), TRAIL receptor 2 (3-fold) and Bak (3-fold). The transcriptional up-regulation of these pro-apoptotic genes occurred as early as 1 h after drug exposure. We also found that IND, but not NS, down-regulated most of the anti-apoptotic genes in the IAP (inhibitor of apoptosis protein) family such as IAP2, XIAP (9-fold), survivin (7-fold) and Bruce (3-fold) (Figure 3). Significant up-regulation and down-regulation of apoptotic-related genes in our study is determined by a 3 or more fold change from control.

Protein analysis

The protein expression of several significantly up-regulated

or down-regulated genes is shown in Figure 4. Both IND and NS treatments up-regulated the pro-apoptotic protein Apaf-1 significantly by 9, 12, and 4 fold at 1, 5, and 18 h, respectively (IND); by 96%, 160% and 102% at 1, 5 and 18 h, respectively (NS) (all $P < 0.01$ *vs* control). In addition, both of these drugs also increased Bak protein expression. However, IND treatment also down-regulated anti-apoptotic proteins such as XIAP by 40% and 22% at 1 and 5 h respectively ($P < 0.05$ *vs* control), while NS treatment did not alter it significantly.

DISCUSSION

The cure for colorectal cancer remains a challenge despite all currently available medical and surgical treatment modalities. The cure rate is particularly low in advanced disease, making chemoprevention an attractive alternative. In recent years, the NSAIDs have attracted a great interest as a potential candidate for the chemoprevention of colorectal cancer. There is now a great body of evidence supporting the role of NSAIDs in tumor chemoprevention by the induction of apoptosis. However, little is known about the exact mechanisms of NSAIDs action on colorectal cancer cells.

With the advent of microarray technology, thousands of genes can be assayed in a single experiment. We applied the microarray technology and focused on apoptosis related genes. By creating a profile of genes linked to a particular biological pathway, the expression of particular genes in a colon cancer cell line treated with NSAIDs, may suggest the activation of specific pathways. Furthermore, comparing the action of a nonselective COX inhibitor *vs* a COX-2-selective inhibitor can reveal mechanistic differences between these two NSAIDs in the induction of apoptotic cell death.

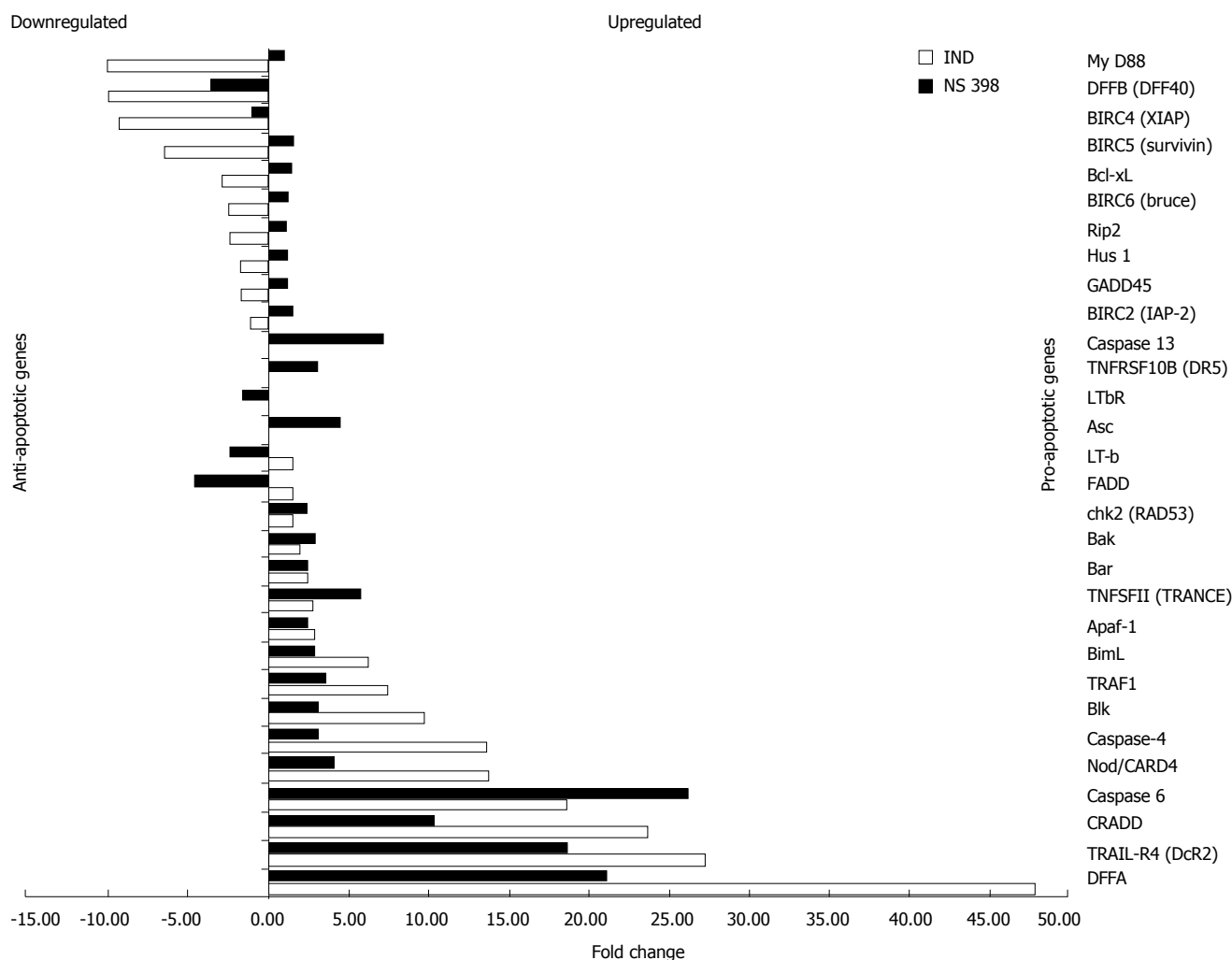


Figure 3 Genes up-regulated and down-regulated by IND and NS treatment identified by cDNA microarray. Up-regulation or down-regulation is expressed as a fold change. Down-regulated genes are shown on the top of the graph in a decreasing order toward mid graph, while up-regulated genes in an increasing order toward the bottom of the graph. Anti-apoptotic genes lie mostly in the upper half while pro-apoptotic genes lie mainly in the bottom half.

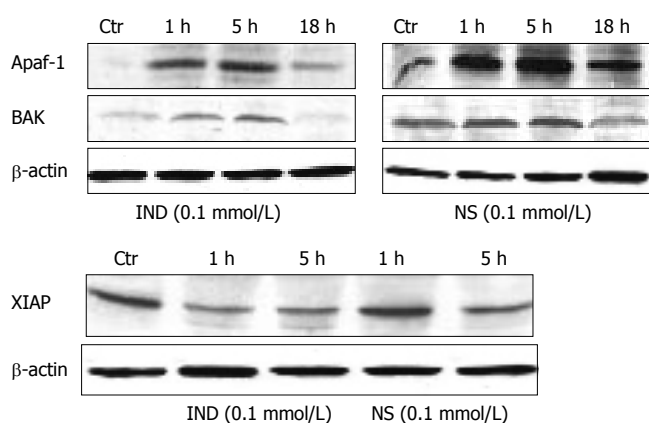


Figure 4 Protein expression by Western blotting of pro-apoptotic genes Apaf-1 and Bak (upper panel), expression of XIAP (lower panel). Blots are representative of triplicate experiments.

Our results suggest that both the nonselective COX inhibitor indomethacin and the COX-2-selective inhibitor NS-398 trigger apoptosis in Caco-2 cells through at least two major well known pathways, an extrinsic, death receptor-transmembrane pathway and an intrinsic, mitochondrial pathway.

The transmembrane pathway of apoptosis involves the tumor necrosis factor (TNF) ligand and receptor superfamily. Members of this family include TNF α , Fas ligand and TRAIL (TNF-related apoptosis-inducing ligand). These ligands when coupling to their respective receptors trigger a number of intracellular events that lead to apoptotic cell death^[25,26]. The final steps of apoptosis induced through this pathway depends on the activation of caspases, cysteine proteases that cleave the aspartate residues^[27,28]. Several members of the TNF family of receptors contain intracellular death domains that interact with other molecules that contain death domains and with caspase recruitment domains (CARD), propagating the apoptotic cascade^[29,30]. We found that both IND and NS-398 up-regulated several genes within the TNF ligand and receptor family such as TNFSF11 (Tumor necrosis factor superfamily member 11), TRAIL-R4, TRAIL-R2. We also detected up-regulation of other genes encoding downstream molecules associated with this pathway such as CRADD (CASP2 and RIPK1 domain containing adaptor with death domain), ASC (Apoptosis-associated speck-like protein containing a CARD), NOD1 (caspase recruitment domain 4), APAF (apoptotic protease activating factor). Our data strongly suggest that NSAIDs

induce apoptosis in Caco-2 cells by up-regulating genes of the TNF ligand and receptor pathway, likely mediated by TNF receptors and TRAIL receptors. This mechanism may explain in part the chemopreventive properties of NSAIDs in colorectal cancer. Several studies have shown promise in using TNF α and TRAIL as potential chemotherapeutic agents in a variety of tumors^[31-34], however, toxicity remains a concern when these agents are used systemically. Our study provides further evidence that NSAIDs likely sensitize colorectal cells to death through TNF and TRAIL receptor. This suggests NSAIDs to be potentially useful as an adjunct chemotherapeutic agent by increasing the therapeutic window of TNF α and TRAIL and decreasing their toxicity.

Our study suggests that NSAIDs also affect the mitochondrial pathway of apoptosis. This intrinsic pathway is usually triggered by chemicals, growth factor deprivation or irradiation. The major steps in this pathway involve the release of cytochrome c with subsequent increase in mitochondrial membrane permeability that is controlled by a variety of pro-apoptotic and anti-apoptotic members of the Bcl-2 family. The release of cytochrome c triggers the assembly of Apaf-1 (Apoptotic protease-activating factor) and pro-caspase 9 to form an apoptosome. The activated caspase 9 then activates other downstream molecules to initiate apoptosis^[35-37]. Several members of the Bcl-2 family were shown to be up-regulated or down-regulated in Caco-2 cells treated with IND and NS. The pro-apoptotic members up-regulated by NSAIDs include Bak (Bcl2-antagonist/killer 1), Blk (B lymphoid tyrosine kinase) and Bim (Bcl2 like 11, apoptosis facilitator). Bcl-x (Bcl2-like 1), an antiapoptotic member, was down-regulated. These data suggest that NSAIDs, both COX nonselective and COX-2 selective likely induce apoptosis *via* the death receptor pathways and the mitochondrial pathway.

There was one major difference in the pattern of genes expressed in Caco-2 cells treated with IND and NS-398. While both IND and NS-398 up-regulated pro-apoptotic genes, IND also down-regulated anti-apoptotic genes, particularly the IAP (inhibitors of apoptosis proteins) family. This family has been described consisting of at least six members and can efficiently inhibit the caspases^[38-40]. In our study, IND treatment of Caco-2 cells caused significantly down-regulated IAP2, XIAP, Survivin and Bruce, while treatment with NS-398 did not affect these genes. This may suggest a mechanistic difference by which non-selective COX and COX-2 specific NSAIDs induce apoptosis. This finding indicates that COX-1 may play a role in the different genetic expression of the IAP family between IND and NS treatments. Further functional studies exploring this difference may contribute to a better understanding of the chemopreventive properties of NSAIDs.

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***Chlamydia pneumoniae* replicates in Kupffer cells in mouse model of liver infection**

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infection, involving cells of the innate immunity such as Kupffer cells, could also trigger pathological immune reactions involving the liver, as observed in human patients with primary biliary cirrhosis.

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Key words: *Chlamydia pneumoniae*; Liver infection; Kupffer cells; Hepatocytes; Culture-isolation; Fluorescence *in situ* hybridization; TNF- α ; Primary biliary cirrhosis

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Abstract

AIM: To develop an animal model of liver infection with *Chlamydia pneumoniae* (*C. pneumoniae*) in intraperitoneally infected mice for studying the presence of chlamydiae in Kupffer cells and hepatocytes.

METHODS: A total of 80 BALB/c mice were inoculated intraperitoneally with *C. pneumoniae* and sacrificed at various time points after infection. Chlamydiae were looked for in liver homogenates as well as in Kupffer cells and hepatocytes separated by liver perfusion with collagenase. *C. pneumoniae* was detected by both isolation in LLC-MK2 cells and fluorescence *in situ* hybridization (FISH). The releasing of TNF- α by *C. pneumoniae* *in vitro* stimulated Kupffer cells was studied by enzyme-linked immunosorbent assay.

RESULTS: *C. pneumoniae* isolation from liver homogenates reached a plateau on d 7 after infection when 6 of 10 animals were positive, then decreased, and became negative by d 20. *C. pneumoniae* isolation from separated Kupffer cells reached a plateau on d 7 when 5 of 10 animals were positive, and became negative by d 20. The detection of *C. pneumoniae* in separated Kupffer cells by FISH, confirmed the results obtained by culture. Isolated hepatocytes were always negative. Stimulation of Kupffer cells by alive *C. pneumoniae* elicited high TNF- α levels.

CONCLUSION: A productive infection by *C. pneumoniae* may take place in Kupffer cells and *C. pneumoniae* induces a local pro-inflammatory activity. *C. pneumoniae* is therefore, able to act as antigenic stimulus when localized in the liver. One could speculate that *C. pneumoniae*

INTRODUCTION

Chlamydia pneumoniae (*C. pneumoniae*) is a common cause of respiratory infections in humans^[1,2], and it is also associated with outcomes other than respiratory disease, including coronary heart disease and myocardial infarction^[3,4]. Systemic disease has also been reported in which *C. pneumoniae* was detected by polymerase chain reaction in lymph nodes and/or liver and spleen^[5]. In addition, recent reports suggest a possible association of *C. pneumoniae* infection in patients with primary biliary cirrhosis (PBC)^[6]. This disease is characterized by the presence of anti-mitochondrial autoantibodies and is considered as an autoimmune disease, although precise etiopathogenetic mechanisms remain unknown^[7]. Infectious agents have been proposed as triggers in susceptible individuals through a mechanism known as molecular mimicry^[8]. It seems therefore possible that antigens from dissociated or alive microbes in Kupffer cells, in other macrophages and lysed cells can trigger immunologically-mediated disorders of the liver such as those observed in PBC^[9].

In intranasally infected mouse, *C. pneumoniae* infection has been shown to spread systemically *via* infected macrophages from the initial infection site, the lung, to other organs, including the spleen and occasionally the liver^[10]. Here, we report on *C. pneumoniae* infection of the liver in intra-peritoneally infected mice and the involvement of Kupffer cells.

MATERIALS AND METHODS

Animal infection

The animals used in the studies were adult (10-11 wk old) Balb/c mice (Morini, S. Polo D'Enza, Italy). Animals anaesthetized with Ketamine, were inoculated intraperitoneally with purified^[11] *C. pneumoniae* elementary body (EB) suspension^[11]. Infected animals received 0.1 mL of organism suspension: the inoculum preparation contained 2.0×10^7 inclusion-forming units (IFU) of EBs. At d 2, 7, 10, and 20 after infection, anaesthetized animals were sacrificed. The protocol was approved by the ethical committee of the University of Bologna.

C. pneumoniae isolation from the liver

Ten animals were tested at each time point: i.e. at 2, 7, 10 and 20 d after infection. The liver was removed, weighed and homogenized in a mortar to obtain a 10% (wt/vol) suspension in cold sucrose phosphate-glutamic acid (SPG) buffer. Tissue suspensions were centrifuged at $300 \times g$ for 10 min at 4°C to remove coarse debris. The clarified homogenates (200 μ L) were inoculated in duplicate onto LLC-MK2 cells (a continuous cell line derived from Rhesus monkey kidney tissue, used to isolate chlamydiae) seeded into plastic individual wells of a 24-well plate, incubated at 37°C for 72 h in chlamydial growth medium (Eagle's minimal essential medium supplemented with 10% heat inactivated fetal calf serum, containing 2 mmol/L glutamine, 5 mg/L glucose and 1 ng/L cycloheximide) and then fixed in methanol. Chlamydial inclusions were visualized by immunofluorescence.

Isolation of Kupffer cells and hepatocytes

To isolate Kupffer cells and hepatocytes, animals, infected as above, were anaesthetized with Ketamine and sacrificed 2, 7, 10 and 20 d after infection: 10 animals were tested at each time. Kupffer cells and hepatocytes were harvested and separated following the procedure of Smedsrød and Pertoft^[12] with minor modifications, as previously described^[13,14]. Briefly, the liver was perfused with 30 mL of calcium- and magnesium-free Hanks' balanced salt solution (BSS) followed by Hanks' (BSS) containing 0.05% collagenase (type IV; Sigma) for 10 min. The liver was then excised and the cells dispersed in calcium- and magnesium-free Hanks' (BSS). The cells were then centrifuged at $50 \times g$ at 4°C for 2 min, in a Beckman J6B centrifuge (Beckman Instrument, Palo Alto, Calif.). The non-parenchymal cell-enriched supernatant was centrifuged at $800 \times g$ for 10 min, the pellet resuspended in 40 mL of PBS, and portions of 10 mL were layered on top of preformed two-step Percoll gradient (the bottom cushion with a density of 1.066 g/mL and an osmolality of 310 mOsm; the overlying cushion with a density of 1037 g/mL and an osmolality of 300 mOsm), and centrifuged at $400 \times g$ for 15 min at 4°C. Purified non-parenchymal cells enriched in Kupffer cells extended throughout the lower Percoll cushion. The pellets consisted of erythrocytes, non-parenchymal liver cells, and other small white cells. The Kupffer cells-enriched fraction was diluted in PBS and centrifuged at $800 \times g$ for 10 min. The resulting pellet was resuspended in culture medium (RPMI 1640 with 10% fetal calf serum) at a concentration of 1.0×10^6 cells/mL. A 0.5-mL portion of cell suspen-

sion was added to 8-well culture plate (Lab-Tek, Nalge Nunc International, Naperville, IL, USA). Kupffer cells were selected by allowing them to adhere for 2 h at 37°C in an atmosphere with 50 mL/L CO₂. After nonadherent cells were removed by gentle washing, adherent cells were incubated in RPMI 1640. More than 95% of adherent cells were esterase-positive. The purity of Kupffer cell preparations was also validated using the FITC labelled anti-mouse F4/80 monoclonal antibody (Tebu-bio, Magenta, Italy). Hepatocytes were obtained by differential centrifugation of the initial suspension of mouse liver cells followed by an additional purification step, consisting of sedimentation of the cells through a single cushion of Percoll (density 1.08 g/mL). The hepatocytes were seeded on fibronectin-coated slides and grown in RPMI 1640 with 10% fetal calf serum. Kupffer cells and hepatocytes were also separated from uninfected animals.

In vitro infection of Kupffer cells and hepatocytes by *C. pneumoniae*

When required, Kupffer cells from uninfected animals were obtained as above described and seeded into 8-well culture plate (1.5×10^5 cells/well) and cultured in RPMI 1640 medium for 24 h at 37°C in an atmosphere with 5% CO₂. Cultures of Percoll-separated hepatocytes were likewise established from uninfected animals by seeding on fibronectin and grown in RPMI 1640 medium, in 8-well culture plate (Lab-Tek). Kupffer cells and hepatocytes were then infected with *C. pneumoniae* EBs (5×10^5 inclusion forming units/mL) and examined for inclusion formation and infectivity at various times after infection by culturing of infected preparations in LLC-MK2 cells.

Detection of viable chlamydiae

To detect the presence of viable chlamydiae in separated Kupffer cells and hepatocytes either from infected animals or from *in vitro* infected Kupffer cells and hepatocytes. Both Kupffer cells and hepatocytes were resuspended in chlamydial growth medium, sonicated and clarified by low speed centrifugation. The supernatant was then inoculated onto LLC-MK2 cells. Chlamydial inclusions were detected by immunofluorescence.

Detection of *C. pneumoniae* by immunofluorescence assay (IFA)

The detection of *C. pneumoniae* by IFA was performed by fixing separated Kupffer cells and hepatocytes or LLC-MK2 cells with methanol for 10 min at room temperature. Inclusions were then visualized using fluorescein-conjugated *C. pneumoniae* species-specific (Chlamydia-cell, Cellabs PTY LTD Brookvale, Australia) monoclonal antibody for 35 min at room temperature. Slides were observed under a Zeiss UV microscope.

Detection of *C. pneumoniae* 16S RNA by fluorescence in situ hybridization (FISH)

The *C. pneumoniae* 16S rRNA detection by FISH was performed by fixing mouse Kupffer cells and hepatocytes within the Lab-Tek culture plates with 2% paraformaldehyde for 30 min at 4°C and prior to FISH, cells were dehydrated with increasing concentrations of ethanol (50%, 80% and 100%). The *Chlamydia*-specific probes used in this

Table 1 Isolation of *C. pneumoniae* from liver homogenates of 40 intraperitoneally infected mice at various days after infection

Days after infection	<i>n</i> (pos)/ <i>n</i> (tested)
2	0/10
7	6/10
10	4/10
20	0/10

Table 2 Detection of *C. pneumoniae* in isolated Kupffer cells and hepatocytes by FISH, IFA and culture, performed in 40 intraperitoneally infected mice at various days after infection

Days after infection	<i>C. pneumoniae</i> by FISH and IFA in:		<i>C. pneumoniae</i> isolation from:	
	Kupffer cells <i>n</i> (pos)/ <i>n</i> (tested)	Hepatocytes <i>n</i> (pos)/ <i>n</i> (tested)	Kupffer cells <i>n</i> (pos)/ <i>n</i> (tested)	Hepatocytes <i>n</i> (pos)/ <i>n</i> (tested)
2	0/10	0/10	0/10	0/10
7	6/10	0/10	5/10	0/10
10	4/10	0/10	4/10	0/10
20	0/10	0/10	0/10	0/10

study have been previously described by Poppert *et al.*^{15]} and deposited in ProbeBase (<http://www.microbial-ecology.de/probebase/index.html>). Cpn-0974 (5'-AAGTCCAG-GTAAGGTCCT-3') was the species-specific 16S rRNA-targeted oligonucleotide probe that was 5' end labelled with Cy3 fluorochrome (Tib Molbiol, s.r.l., Genova, Italy) giving a red-orange signal. For Kupffer cells and hepatocytes hybridization, a 10 µL aliquot of the hybridization buffer (0.9 mol/L NaCl, 20 mol/L Tris-HCl pH 8, 0.01% SDS and from 0% to 30% formamide) containing 5 pmol of each fluorescent probe was applied to each well of Lab Tek culture plate. After 1 h of incubation in a moist chamber at 46°C in the dark, the slides were washed for 15 min in pre-heated washing buffer containing 20 mmol/L Tris-HCl pH 8, from 0.9 mol/L to 0.15 mol/L NaCl (depending on formamide concentration) and 0.01% SDS. The slides were air dried and mounted with Citifluor AF1 (Citifluor Ltd, London, United Kingdom). The slides were viewed under an epi-fluorescence microscope (Eclipse E600, Nikon) equipped with a super high pressure mercury lamp and Plan Fluor DLL 10 ×, 40 ×, 100 × objectives. Epi-fluorescence filter G-2A was used to analyse Cy3 signal at a magnification of 1000 ×. Photomicrographs were taken using a DXM-1200 digital camera (Nikon, Japan) and image processing was performed with ACT-1 for DXM-1200 software.

TNF-α production by Kupffer cells

To evaluate the TNF-α induction in Kupffer cells infected by *C. pneumoniae* *in vitro*, Kupffer cells, purified as above reported, were added (5×10^5) to each well of an 8-well culture plate (Lab-Tek). After 2 h at 37°C, non-adherent cells were removed by washing three to five times with RPMI 1640 supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine. Cells were stimulated for 6 h with either LPS (10 mg/L) (Sigma) or viable *C. pneumoniae* at a ratio of 100 EBs/cell. The supernatants were then harvested and tested by enzyme-linked immunosorbent assay (ELISA) kit for TNF-α (Bender MedSystems GmbH, Wien, Austria),

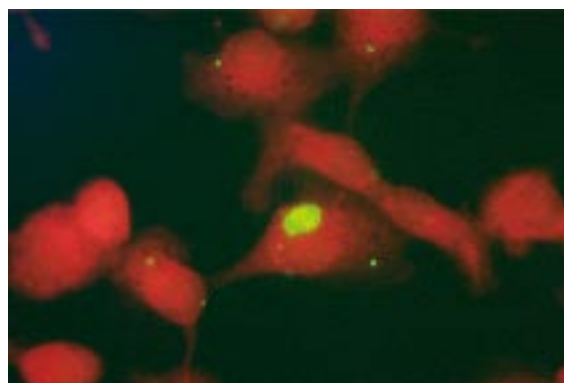


Figure 1 IFA for the detection of *C. pneumoniae* antigens in separated Kupffer cells. 1000 ×. Kupffer cells separated from intraperitoneally infected mice sacrificed 7 d after infection were tested by immunofluorescence assay (IFA) with specific fluorescein-conjugated monoclonal antibodies. A positive Kupffer cell is clearly evident for the presence of an apple-green intracytoplasmic bacterial inclusion. The inclusion represents an intracytoplasmic bacterial microcolony.

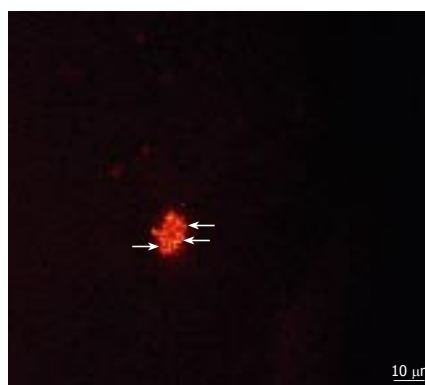


Figure 2 FISH for *C. pneumoniae* 16S rRNA in separated Kupffer cells. *In situ* hybridization was performed on Kupffer cells separated from intraperitoneally infected mice, sacrificed 7 d after infection. An infected Kupffer cell is evidenced with Cpn-0974 specific probe: the granular inclusion stained red.

according to the manufacturer's protocol. When required, preincubation of material with polymyxin B (10 mg/L) (Sigma) was used to abrogate the effect of LPS.

RESULTS

Four groups of infected animals (10 for each group) were sacrificed 2, 7, 10 and 20 d following intraperitoneal infection by *C. pneumoniae* and isolation of viable chlamydiae was performed from liver homogenates. The peak of *C. pneumoniae* isolation from the liver was obtained on d 7 after infection, thereafter the infectivity decreased, all the animals became negative by d 20 of infection (Table 1).

In order to verify whether *C. pneumoniae* was able to infect *in vivo* Kupffer cells and/or hepatocytes, purified liver cells were tested by IFA, to detect chlamydial antigens and by FISH to detect chlamydial rRNA. Four further groups of infected animals (10 for each group) were sacrificed 2, 7, 10, and 20 d after infection, respectively, then isolated Kupffer cells and hepatocytes were analysed. Positive Kupffer cells were observed in preparations obtained on d 7 and 10 (Table 2), both by IFA and FISH, thus confirming the results obtained by culture. Kupffer cells purified 20 d after infections were negative. Positive cells showed

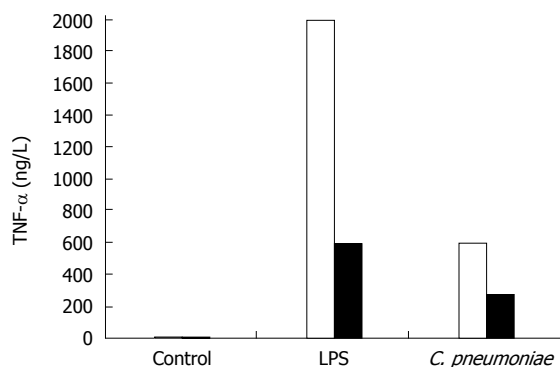


Figure 3 TNF- α release by Kupffer cells following *in vitro* exposure to alive *C. pneumoniae*. Kupffer cells were stimulated for 6 h with alive *C. pneumoniae*. Control LPS was used at 10 mg/L. Stimulation was performed with (black) or without (white) polymyxin B at concentration of 10 mg/L.

cytoplasmic apple-green inclusions by IFA (Figure 1) and red granular inclusions by FISH (Figure 2). The number of positive cells was 10/100 in preparations obtained 7 d after infection, and 5/100 in preparations obtained 10 d after infection. The preparations from animals sacrificed 20 d after infection were negative. The passage of the content of Kupffer cells obtained from IFA- and FISH-positive animals induced chlamydial inclusion formations in LLC-MK2 cells. Hepatocytes were always negative by IFA and FISH and by culture in LLC-MK2 cells.

The *in vitro* infection of purified mouse Kupffer cells and hepatocytes by *C. pneumoniae* was followed by FISH and by culture: Kupffer cells were positive for the presence of inclusions detectable by 12 h of infection. Sub-culturing Kupffer cells in LLC-MK2 mono-layers three days after infection allowed re-isolation of *C. pneumoniae*. Hepatocytes infected *in vitro* by *C. pneumoniae* and studied by IFA and FISH was negative 12, 24, and 48 h after infection. Sub-culturing hepatocytes in LLC-MK2 cells three days after infection did not allow re-isolation of *C. pneumoniae*, demonstrating that *C. pneumoniae* is unable to infect hepatocytes.

In vitro stimulation of Kupffer cells by alive *C. pneumoniae* elicited cellular responses resulting in the production of TNF- α (Figure 3). Pretreatment of chlamydiae with polymyxin B, which binds and inactivates LPS^[16], substantially diminished the ability of bacteria to stimulate Kupffer cells (Figure 3).

DISCUSSION

Chlamydiae are obligate intracellular bacteria parasitizing eukaryotic cells. Chlamydiae replicate in the cytoplasm of infected cells within an inclusion that does not fuse with lysosomes^[17]. Within the genus *Chlamydia*, various species preferentially infect different target cells and cause a variety of diseases all ultimately due to inflammatory responses. In particular, *C. pneumoniae* has been associated with respiratory human infections and with the development of atherosclerosis and cardiovascular disease^[18-21]. *C. pneumoniae* has been also associated with sarcoidosis and it was detected in 4 of 38 liver biopsies from autopsy specimens of patients with sarcoidosis^[22]. In a rabbit model of *C. pneumoniae* in-

fection, three of 10 infected rabbits had evidence of *C. pneumoniae* elementary bodies in their livers by immunocytochemical staining and *C. pneumoniae* was cultured in two of them^[23]. In a hamster model of *C. pneumoniae* infection, similarly *C. pneumoniae* was isolated from the liver of the infected animals^[24]. These studies altogether indicate that *C. pneumoniae* can potentially persist in liver tissues.

More recently, Abdulkarim *et al*^[6] reported the presence of *C. pneumoniae* antigens and rRNA in liver tissue of patients with end-stage primary biliary cirrhosis, suggesting a potential role of *C. pneumoniae* in the etiology and pathogenesis of the disease, in contrast with serological data by Liu *et al*^[25] that do not support the concept that *Chlamydia pneumoniae* can be a triggering or causative agent in PBC.

To get further insight into a possible role of *C. pneumoniae* in liver related pathologies, evidence was to be produced as to the possible localization and/or replication of *C. pneumoniae* in parenchymal and not parenchymal liver cells, mainly in Kupffer cells, where chlamydiae could act as a trigger for cellular immunologically-mediated alterations. Kupffer cells constitute the largest population of fixed tissue macrophages found in the body, and blood-clearance and elimination of bacteria taken up by the liver are widely attributed to Kupffer cells. Sometimes microorganisms can survive or even multiply in Kupffer cells, as observed in *Leishmania* and *Salmonella typhi* infections in mice and humans^[26,27]. In this study, we have described a mouse model of *C. pneumoniae* infection where the organism demonstrated the ability to infect and to multiply in Kupffer cells both *in vivo* and *in vitro*, and to trigger the release of the proinflammatory cytokine TNF- α , by these macrophages. In our animal model, mice eliminated *C. pneumoniae* by d 20 of infection and a chronic infection was not established. However, it is well known that persistence, for as yet unpredictable reasons, is a frequent case of human chlamydial infections^[28].

PBC is a chronic liver inflammatory disease whose etiology and pathogenesis remain still unknown. A wide range of data suggest an autoimmune pathogenesis for the disease^[29,30], mostly based on the presence of anti-mitochondrial autoantibodies and autoreactive T cells directed against autoantigens. Despite the fact that the autoimmune reaction is directed against ubiquitous mitochondrial autoantigens, the ensuing damage involves primarily biliary epithelial cells^[7]. Infectious agents have been proposed as triggers in susceptible individuals through a mechanism known as molecular mimicry^[8]. However, over the past years several studies failed to demonstrate a specific chronic microbial^[8,31], viral^[32,33] or bacterial^[34-36] infection. Recently, it has been proposed that an aberrant innate immune response to infections has the potential to initiate the development of autoimmunity^[37]. The ability of *C. pneumoniae* to survive and to replicate in Kupffer cells, as documented in this study, demonstrates the ability of this bacterium to escape, under certain conditions, the defence mechanisms of natural immunity. This characteristic can favour, as suggested by Liu *et al*^[25], the production of highly immunogenic substrates and/or provide an inflammatory microenvironment to enhance the self-reactivity of pathogenic T lymphocytes, or can activate pre-existing autoreactive cellular repertoire.

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BASIC RESEARCH

Peroxisome proliferator-activated receptor γ agonist reduces the severity of post-ERCP pancreatitis in rats

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Abstract

AIM: To determine the effects of prophylactic peroxisome proliferator-activated receptor (PPAR γ) agonist administration in an experimental model of post-endoscopic retrograde cholangiopancreatography (post-ERCP) acute pancreatitis.

METHODS: Post-ERCP pancreatitis was induced in male Wistar rats by infusion of contrast medium into the pancreatic duct. In additional group, rosiglitazone, a PPAR γ agonist, was administered 1 h before infusion of contrast medium. Plasma and pancreas samples were obtained 6 h after the infusion.

RESULTS: Infusion of contrast medium into the pancreatic duct resulted in an inflammatory process characterized by increased lipase levels in plasma, and edema and myeloperoxidase activity (MPO) in pancreas. This result correlated with the activation of nuclear factor κ B (NF κ B) and the inducible NO synthase (iNOS) expression in pancreatic cells. Rosiglitazone reduced the increase in lipase and the level of edema and the increase in myeloperoxidase as well as the activation of NF κ B and iNOS expression.

CONCLUSION: A single oral dose of rosiglitazone, given 1 h before post-ERCP pancreatitis induction is effective in reducing the severity of the subsequent inflammatory process. The protective effect of rosiglitazone was associated with NF κ B inhibition and the blockage of leukocyte infiltration in pancreas.

INTRODUCTION

Acute pancreatitis is one of the major and serious complications after diagnostic or therapeutic endoscopic retrograde cholangiopancreatography (ERCP). Despite the technical improvements of recent years and the experience of endoscopists, the incidence has not decreased and it ranges from 1% to 10% of patients^[1]. The most severe forms of pancreatitis, with pancreatic necrosis, multi-organ failure and even death, occurs in 0.3%-0.6% of patients, but a silent increase in serum pancreatic enzymes could be observed in up to 70% of patients^[2]. The triggering mechanism of the inflammatory response remains unclear and different pharmacological agents has been tested to prevent post-ERCP pancreatitis including anti-inflammatory steroids, somatostatin analogs, heparin, protease inhibitors and anti-inflammatory cytokines^[3-6]. Only few drugs showed some efficacy to prevent post-ERCP pancreatitis like recently nitrate therapy by decreasing the pancreatic ductal pressure or diclofenac by lowering inflammatory process^[7,8]. Therefore, the availability of effective drugs and strategy of chemoprevention remain as unsettled points in the pharmacological prophylaxis of post-ERCP pancreatitis^[5-8], leading to the apparition of endoscopic procedure such as pancreatic sphincterotomy to try to decrease the risk of post-ERCP pancreatitis^[9].

Recent evidences indicate an important role for the peroxisome proliferator-activated receptors (PPARs) in the regulation of both inflammation and lipid metabolism^[10]. In particular, it has been reported, using an experimental model of cerulein-induced pancreatitis, that the pancreatic inflammation and tissue injury was markedly reduced by the administration of PPAR γ agonists^[11]. PPAR γ is a member of the nuclear hormone receptor superfamily originally reported to be expressed at high levels in

adipose tissue and to play a critical role in adipocyte differentiation, glucose metabolism and lipid storage. In an experimental model of intestinal ischemia-reperfusion, a more severe injury was observed in PPAR γ -deficient mice and protection against local and remote tissue injury in mice treated with a PPAR γ -activating ligand^[12]. Then, it has been demonstrated that PPAR γ ligands can inhibit the inflammatory response by decreasing IL-6, IL-1 β , TNF α and the inducible NO synthase (iNOS) by interfering with nuclear factor κ B (NF κ B) and AP1^[13,14]. The aim of this study was to investigate the efficacy of prophylactic PPAR γ agonist treatment in reducing the pancreatic damage in an experimental post-ERCP acute pancreatitis model.

MATERIALS AND METHODS

Reagents

Reagents for SDS-PAGE and nitrocellulose membranes were from Amersham Pharmacia (Buckinghamshire, England). Antibodies against p65, PPAR γ and Histone H1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), antibody against iNOS was obtained from BD Transduction (Heidelberg, Germany), antibody against β -actin and the secondary antibody linked to horseradish peroxidase were from Sigma Chemicals (St Louis, MO). The following reagents were obtained from Sigma Chemicals (St Louis, MO): NaVO₃, NaF, Nonidet P40, ethidium bromide, Hexadecyltrimethylammonium bromide, Tetramethylbenzidine, DMSO, H₂O₂.

Post-ERCP pancreatitis model

Male Wistar rats (250–300 g) were used for all experiments. Animals were housed in light-dark cycle regulated, air conditioned (23°C) and air humidity (60%) animal quarters, given free access to drinking water and standard food pellets until 12 h prior to the experiment, at which point food was withdrawn. Animal care was in compliance with the European Community (Directive 86/609/EEC) for the use of experimental animals and the institutional committee of animal care and research approved it. Rats were anaesthetized with ip injection of sodium pentobarbital (10 μ L/kg). The biliopancreatic duct was cannulated through the duodenum and the hepatic duct was closed by a small bulldog clamp. Post-ERCP pancreatitis was induced by retrograde infusion into the biliopancreatic duct of low osmolarity contrast medium Meglumine/Sodium Ioxaglate (Hexabrix 320) in a volume of 10 μ L/kg using a Harvard '22' infusion pump (Harvard Instruments, Edenbridge, UK). Control animals were subjected to anesthesia and laparotomy^[15].

Experimental design

In the first set of experiments, we evaluated the severity of pancreatic damage and tissue inflammation after the infusion of contrast medium into the pancreatic duct. For this purpose, rats ($n = 6$ for each group) were sacrificed at 0, 3, 6 and 24 h after infusion and samples of pancreatic tissue and plasma were obtained, immediately frozen and maintained at -80°C until assayed.

In a second series of experiments, a PPAR γ agonist (Rosiglitazone, AVANDIA[®] GlaxoSmithKline, Brentford,

UK) was administered (10 mg/kg intragastric bolus) 1 h before infusion of contrast medium ($n = 8$ for each group)^[11]. Samples of plasma and pancreas were obtained 6 h after infusion.

Edema

The extent of pancreas edema was assayed by measuring tissue water content. Freshly obtained samples of pancreas were weighted on aluminum foil, dried for 24 h at 95°C and reweighed. The difference between wet and dry tissue weight was calculated and expressed as a tissue wet: dry mass ratio.

Myeloperoxidase activity

Neutrophil infiltration was assessed by measuring myeloperoxidase (MPO) activity. Myeloperoxidase was measured photometrically with 3,3',5,5'-tetramethylbenzidine as a substrate^[16]. Samples were macerated with 5 g/L hexadecyltrimethylammonium bromide in 50 mmol/L phosphate buffer pH 6.0. Homogenates were then disrupted for 30 s using a Labsonic (B.Braun) sonicator at 20% power and submitted to three cycle of snap freezing in dry ice and thawing before a final 30 s sonication. Samples were incubated at 60°C for 2 h and then spun down at 4000 g for 12 min. Supernatants were collected for myeloperoxidase assay. Enzyme activity was assessed photometrically at 630 nm. The assay mixture consisted of 20 μ L supernatant, 10 μ L tetramethylbenzidine (final concentration 1.6 mmol/L) dissolved in DMSO and 70 μ L H₂O₂ (final concentration 3.0 mmol/L) diluted in 80 mmol/L phosphate buffer pH 5.4. An enzyme unit is defined as the amount of enzyme that produces an increase of 1 absorbance unit per minute.

NF κ B and PPAR γ DNA binding

Binding of NF κ B p65 subunit to the NF κ B binding consensus sequence 5'-GGGACTTTC-3' and binding of PPAR γ to the PPRE binding consensus sequence 5'-AACTAGGTCAAAGGTCA-3' were measured with the ELISA-based TransAM kits (Active Motif, Carlsbad, CA) using tissue nuclear extracts. This assay is performed in 96-well plates coated with an oligonucleotide containing the binding consensus sequence. The active forms in nuclear extracts can be detected using specific Abs for epitopes that are accessible only when the subunits are activated and bound to its target DNA. Specificities were checked by measuring the ability of soluble wild-type oligonucleotides to inhibit binding.

Western blot

Pancreatic tissue was lysed by using the Nuclear Extract Kit from Active Motif (Carlsbad, CA) following the manufacturer conditions for preparation of cytoplasmic and nuclear extracts. SDS-PAGE was performed using 100 g/L or 120 g/L acrylamide gels. Proteins were electrotransferred to nitrocellulose membrane and probed with primary Ab (anti-p65, 1/1000; anti-PPAR γ , 1/200; anti-iNOS, 1/400; anti- β -actin, 1/400; anti-Histone H1, 1/500). The membranes were incubated with corresponding horseradish peroxidase-linked secondary Ab, washed and subsequently incubated with ECL reagents

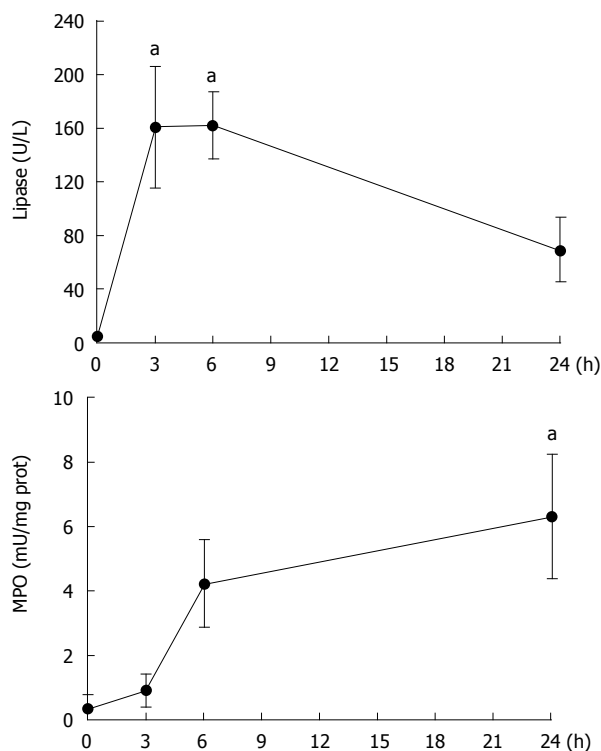


Figure 1 Plasma lipase and pancreas MPO activity after retrograde infusion of contrast media. (^a $P < 0.05$ vs $t = 0$).

from Amersham Pharmacia (Buckinghamshire, England) before exposure to high performance chemiluminescence films. Gels were calibrated using Bio-Rad standard proteins (Hercules, CA) with markers covering a 7-240 kDa range.

Protein measurement

Total protein concentration in homogenates was determined using a commercial kit from BioRad (Munich, Germany).

Lipase

Plasma lipase was determined by using commercial kits from Randox (Antrim, UK), according to the supplier's specifications.

Histological study

Pancreatic tissue samples were taken and fixed in 40 g/L neutral buffered formaldehyde solution, paraplast-embedded, cut into 5 μ m sections and stained with hematoxylin-eosin for light microscopy. Two different observers evaluated randomly ten fields from each animal and cell infiltration was recorded blindly on photomicrographs.

Statistical analysis

Data have been expressed as mean \pm SE. Means of different groups were compared using a one-way analysis of variance. Tukey's multiple comparison test was performed for evaluation of significant differences between groups. Differences were assumed to be significant when $P < 0.05$.

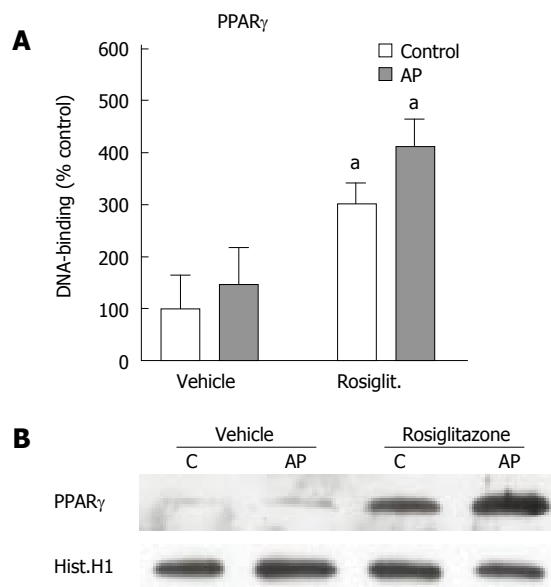


Figure 2 PPAR γ activation. **A:** PPAR γ DNA binding activity of pancreas nuclear extracts expressed as % of control activity. No significant differences were observed on PPAR γ binding to DNA after pancreatitis induction. By contrast, PPAR γ binding to DNA is strongly induced by rosiglitazone in both control and pancreatitis groups. ^a $P < 0.05$ Rosiglitazone-treated vs vehicle-treated groups. **B:** Western blot of nuclear PPAR γ confirmed the nuclear translocation of PPAR γ protein after rosiglitazone administration in both control and pancreatitis groups. Western blot was representative of three different experiments.

RESULTS

ERCP-induced pancreatic damage

The evolution of pancreatic damage was evaluated by measuring plasma lipase and tissue MPO activity at different time points after pancreas infusion of contrast medium (Figure 1). A rapid and significant increase was observed in plasma lipase activity that achieved a peak between 3 and 6 h after surgery. By contrast, MPO activity was not increased until 6 h after infusion and remained increased until the end of experiment (24 h). Since in this model the inflammation required 6 h to be established, we selected this time point for the rest of experiments.

Treatment with rosiglitazone induced pancreatic activation of PPAR γ

To evaluate the efficacy of rosiglitazone to induce PPAR γ activation in the pancreatic tissue, we measured, on nuclear extracts, the levels of DNA binding activity to an immobilized oligonucleotide containing the PPPE sequence. Binding activity was significantly increased in rosiglitazone-treated animals (Figure 2A). By contrast, in non-treated animals, ERCP-induced pancreatitis was not associated with changes in pancreatic PPAR γ activity. These results were confirmed by western blot analysis of the translocation of PPAR γ into the nuclear fraction upon rosiglitazone treatment (Figure 2B).

Rosiglitazone reduced the ERCP-induced pancreatic damage

Both tissue edema and plasma lipase activity showed

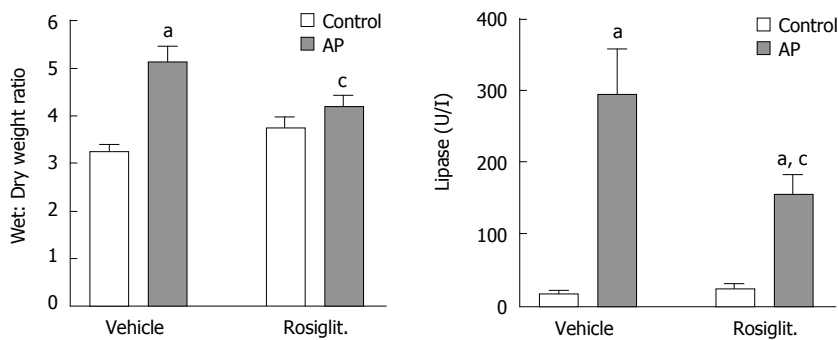


Figure 3 Pancreatic tissue edema and plasma lipase activity 6 h after contrast medium infusion. Retrograde administration of contrast medium induced increases in edema and lipase activity. These increases were partially prevented by pre-treatment of rosiglitazone. ^a $P < 0.05$ AP vs their corresponding control; ^c $P < 0.05$ rosiglitazone-treated AP group vs vehicle-treated AP group.

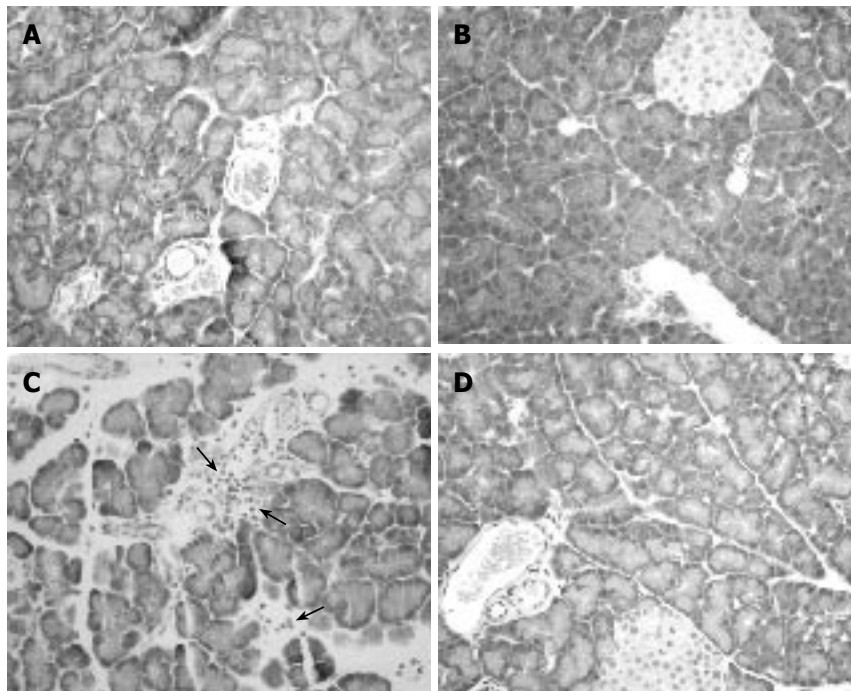


Figure 4 Histological examination of the pancreas (x 200). **A:** Control pancreas showed normal acinar structure; **B:** No morphological changes were observed after rosiglitazone administration in control animals; **C:** Experimental ERCP-induced pancreatic damage reflected in interlobular and interacinar edema and areas of leukocyte infiltration (arrows). Acinar necrosis was not observed; **D:** Rosiglitazone pre-treatment before contrast medium infusion resulted in a reduced edema and absence of leukocyte cell infiltration.

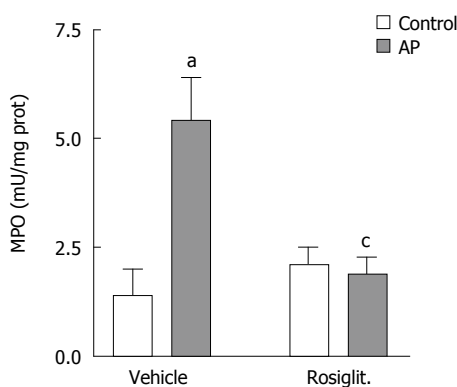


Figure 5 Myeloperoxidase activity in pancreas. MPO activity was significantly increased in post-ERCP induced pancreatitis in comparison with vehicle. Rosiglitazone treatment prevented this increase. ^a $P < 0.05$ AP vs control; ^c $P < 0.05$ rosiglitazone-treated AP group vs Vehicle-treated AP group.

increased levels 6 h after contrast media infusion (Figure 3). Pre-treatment with rosiglitazone significantly reduced these increases, but not to control levels. Histological findings also showed a clear reduction on pancreatic interlobular edema (Figure 4). No acinar necrosis was observed after contrast-media infusion.

ERCP-induced inflammatory response was inhibited by rosiglitazone

When measuring the MPO activity, we observed that the increase induced by contrast medium infusion was completely abrogated by pre-treatment with rosiglitazone (Figure 5). This result was confirmed by histological results. In ERCP group, areas of intense cell infiltration with extravasation of leukocytes to the interacinar space were observed (Figure 4C). Treatment with rosiglitazone completely prevented the infiltration of leukocytes (Figure 4D).

Rosiglitazone prevented the ERCP-induced NF κ B-activation

In order to evaluate the possible involvement of NF κ B on this anti-inflammatory effect, we measured the levels of p65 DNA binding activity in pancreatic nuclear extracts. Results indicated that infusion of contrast media into the pancreatic duct induced a significant activation of NF κ B (Figure 6A). This increase was completely prevented by pre-treatment of rosiglitazone. The effect of the PPAR γ agonist on NF κ B was confirmed by detecting, by western blot, the presence of p65 subunit of NF κ B into the nuclear fraction (Figure 6B). In the ERCP group, p65

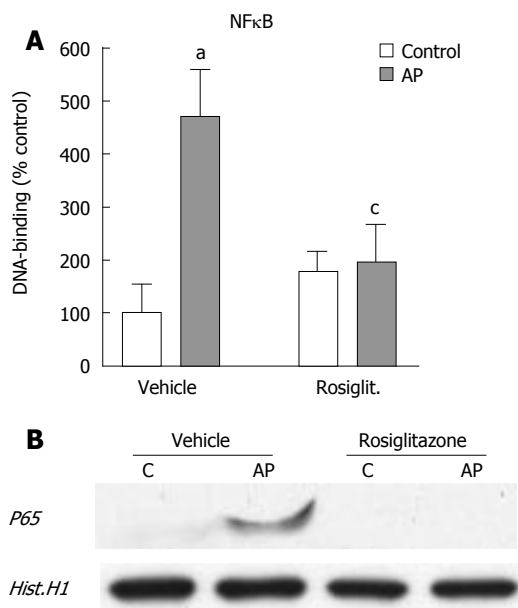


Figure 6 NFκB activation. **A:** NFκB DNA binding activity of pancreas nuclear extracts expressed as % of control p65 activity. Post-ERCP induced pancreatitis resulted in increased levels of p65 binding activity. This increase was inhibited by rosiglitazone pre-treatment. * $P < 0.05$ AP vs Control; * $P < 0.05$ rosiglitazone-treated AP group vs vehicle-treated AP group; **B:** Western blot of nuclear p65 confirmed the nuclear translocation of p65 protein during ERCP-induced pancreatitis. This nuclear translocation was not observed after rosiglitazone administration. Western blot was representative of three different experiments.

translocated into the nucleus and this translocation was prevented by pre-treatment of rosiglitazone.

Rosiglitazone prevented ERCP-induced expression of iNOS

Finally, similar result was found when determining the expression of iNOS, an NFκB-dependent enzyme (Figure 7). In ERCP group, iNOS was strongly induced, and pre-treatment with rosiglitazone prevented this increase. Interestingly, the enzyme, that is undetectable in control animals showed a weak expression in control animals treated with rosiglitazone.

DISCUSSION

Since pancreatitis represents the most common complication after ERCP, different drugs, including IL-10, gabexate mesylate, heparin or somatostatin, nitrate derivatives or diclofenac have been tested to reduce the incidence and severity of post ERCP-pancreatitis. Many of these studies, despite the use of randomized procedures were criticized because of non reproducible results^[3]. In addition, the multifactorial etiology and pathophysiology of post-ERCP pancreatitis needs to be taken into account. Simplified procedures and the absence of adverse effects are required to deliver prophylactic treatment for post-ERCP pancreatitis. Our study shows that administration of a PPARγ agonist in a single oral dose before starting ERCP decreases the severity of the inflammatory reaction triggered by this procedure. Evidence has been accumulated indicating that PPARγ plays a role modulating the inflammation. Several studies have demonstrated that the use of PPARγ ligands inhibits the intensity of the

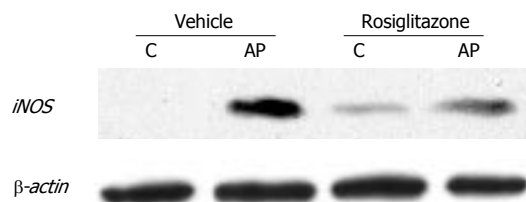


Figure 7 Inducible NO synthase (iNOS) expression. Western blot of cytoplasmatic iNOS showed increased expression of this protein during post-ERCP induced pancreatitis. This increase was reduced by rosiglitazone pre-treatment. Results are representative of three separate experiments.

inflammatory response in different processes including colitis^[17], adjuvant-induced arthritis^[14], and cerulein-induced pancreatitis^[11]. In vitro, the expression of inflammatory mediators such as TNFα, IL-1β, IL-6, iNOS or MMP-9^[13,18] could be inhibited by PPARγ ligands. These findings have raised the possibility that these agents could be useful for the treatment of the inflammatory disorders.

Our results indicate that administration of rosiglitazone before experimental ERCP completely prevented the inflammatory response in the pancreas, reflected in a reduced MPO activity and the lack of leukocytes infiltrate observed in pancreas. By contrast, increases in lipase plasma activity and edema were only partially prevented by rosiglitazone. This fact was not unexpected, since rosiglitazone prevents the activation of the inflammatory response, but has no effect on the mechanical damage related with changes in osmolarity or increased intraductal pressure. Pancreatic damage results from both mechanical and inflammatory processes associated with intraductal activation of pancreatic proenzymes. Nitrate derivatives or pancreatic stenting have been proposed to increase pancreatic out-flow, diclofenac or IL-10 following inflammatory response and somatostatin to decrease the intraductal concentration of pancreatic proenzymes^[5-9]. Rosiglitazone was supposed to act only on the inflammatory-related increases in lipase and edema.

It is known that the activation of PPARγ results in a reduction of the inflammatory response due to its inhibitory effect on main inflammatory signal transduction pathways, in particular, NFκB. It has been reported, in several experimental models of pancreatitis, that pancreatic damage was associated with increased nuclear translocation of NFκB dimers p65/p50 that trigger the transcription and generation of a broad spectrum of inflammatory mediators by pancreatic cells^[19]. These mediators, including cytokines, chemokines and adhesion molecules, generate a microenvironment that promotes the recruitment and activation of inflammatory cells and contributes to increasing the extension of the pancreatic damage. Consequently, we have evaluated the involvement of NFκB in the observed anti-inflammatory effect of PPARγ in post-ERCP pancreatitis. For this purpose we have measured the nuclear translocation and DNA binding of p65, the key component of NFκB. The results indicate that nuclear translocation of p65 was significantly increased after experimental ERCP and this increase was completely prevented by pre-treatment of rosiglitazone. This inhibition could explain the lack of inflammatory

infiltrate observed in pancreas, since NF κ B activation is a requisite for the generation of the main pro-inflammatory mediators involved in pancreatitis.

Similar results were observed when measuring the expression of iNOS in pancreatic tissue. This enzyme was induced mainly in activated leukocytes in order to generate nitric oxide at a cytotoxic concentration as a part of the bactericidal mechanism of these cells. The presence of iNOS in pancreas in post-ERCP pancreatitis confirms that an intense inflammatory process was triggered. Since the synthesis of this enzyme is strongly dependent on NF κ B, it is not a surprise that rosiglitazone treatment downregulates the expression of iNOS. On the other hand, the reduced levels of iNOS could also reflect the lack of cell infiltration that occurs under these conditions.

The blockage of NF κ B activation and the resulting inhibition in the NF κ B-dependent mediators is of importance not only for the local inflammation, but also in order to prevent the release into the circulatory bloodstream of pro-inflammatory cytokines that could trigger a systemic inflammatory response. Although this occurs in a reduced percentage of patients, the severity of the process justifies the use of prophylactic measures to prevent it despite the failure or the weakness of many previous series.

In conclusion, despite the general limitations of all the animal models relative to clinical setting and the multifactorial etiology of post-ERCP pancreatitis, these results suggest that rosiglitazone and other PPAR γ agonists are potential new therapeutic agents for the prevention of post-ERCP-induced acute pancreatitis. It could be administered in an oral dose to the patients, shortly before the ERCP. However, further studies are needed to determine the proper dose and time-point of administration in human patients.

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BASIC RESEARCH

Attenuation of dextran sodium sulphate induced colitis in matrix metalloproteinase-9 deficient mice

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Abstract

AIM: To study whether matrix metalloproteinase-9 (MMP-9) is a key factor in epithelial damage in the dextran sodium sulphate (DSS) model of colitis in mice.

METHODS: MMP-9-deficient and wild-type (wt) mice were given 5% DSS in drinking water for 5 d followed by recovery up to 7 d. On d 5 and 12 after induction of colitis, gelatinases, MMP-2 and MMP-9, were measured in homogenates of colonic tissue by zymography and Western blot, whereas tissue inhibitor of metalloproteinases (TIMPs) were measured by reverse zymography. The gelatinolytic activity was also determined in supernatants of polymorphonuclear leukocytes (PMN) isolated from mice blood. Moreover, intestinal epithelial cells were stimulated with TNF- α to study whether these cells were able to produce MMPs. Finally, colonic mucosal lesions were measured by microscopic examination.

RESULTS: On d 5 of colitis, the activity of MMP-9 was increased in homogenates of colonic tissues (0.24 ± 0.1 vs 21.3 ± 6.4 , $P < 0.05$) and PMN from peripheral blood in wt (0.5 ± 0.1 vs 10.4 ± 0.7 , $P < 0.05$), but not in MMP-9-deficient animals. The MMP-9 activity was also up-regulated by TNF- α in epithelial intestinal cells (2.5 ± 0.5 vs 14.7 ± 3.0 , $P < 0.05$). Although colitis also led to increase of TIMP-1 activity, the MMP-9/TIMP-1 balance remained elevated. Finally, in the MMP-9-deficient colitic mice both the extent and severity of intestinal epithelial

injury were significantly attenuated when compared with wt mice.

CONCLUSION: We conclude that DSS induced colitis is markedly attenuated in animals lacking MMP-9. This suggests that intestinal injury induced by DSS is modulated by MMP-9 and that inhibition of this gelatinase may reduce inflammation.

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Key words: Matrix metalloproteinases; MMP-9-deficient; Dextran sodium sulphate; Inflammatory bowel disease; Experimental colitis

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INTRODUCTION

Matrix metalloproteinases (MMPs) comprise a group of zinc and calcium-dependent endopeptidases that exhibit differential proteolytic activity against extracellular matrix (ECM) proteins. Based on substrate specificity, MMPs have been classically divided into collagenases, gelatinases (MMP-9 and MMP-2) and stromelysins. These proteinases are secreted as latent enzymes that require proteolytic cleavage for activation. However, a subset of MMPs, known as membrane-type MMPs (MT-MMPs) are not secreted but instead remain attached to cell surfaces and activate secreted MMPs^[1,2]. The MMP activity is tightly controlled by specific endogenous inhibitors of these enzymes (TIMPs), which complex with MMPs. Four TIMPs have been described until now and TIMP-1 seems to be the most important endogenous inhibitor, which binds to activated interstitial collagenase and gelatinases^[3-5].

Ulcerative colitis (UC) is a chronic inflammatory disease affecting primarily the distal colon and rectum of young adults and its etiology still remains unclear. Degradation and remodeling of the ECM is increasingly implicated in the pathogenesis of several inflammatory disorders,

such as periodontal disease and rheumatoid arthritis^[6-8]. In physiological conditions, MMPs act as part of the normal connective tissue turnover. However, MMPs released by diverse cells in response to several cytokines during inflammation, may lead to excess degradation of ECM and tissue injury. Previous studies have found both increased activity and expression of MMPs in colonic tissues of patients with inflammatory bowel disease (IBD)^[9-13]. Moreover, MMP-9 may be a key factor responsible for accelerated breakdown of ECM in UC, because it was demonstrated that MMP-9 is abundantly expressed in patients with UC compared with controls^[9]. Hence, MMP inhibition has emerged as a potential therapeutic approach in colonic inflammatory disorders^[14-16]. Furthermore, we have previously demonstrated a significant up-regulation of MMP-9 expression and activity in a rat model of colitis induced by dextran sulphate sodium (DSS). In this experimental model, a synthetic MMP inhibitor (CGS-27023-A) significantly reduced the extent and severity of tissue injury^[17]. However, this compound is a broad spectrum MMP inhibitor and the relative contribution of MMP-9 in this experimental model still remains unclear.

In the present investigation, we sought to determine whether MMP-9 extinction could alter intestinal damage in a mouse model of colitis induced by DSS. This model exhibits clinical and morphological features resembling human UC, including diarrhea and rectal bleeding, diffuse lesions circumscribed to the mucosa, and predominance of distal involvement of the large intestine^[18]. Our findings support the conclusion that MMP-9 modulates colonic mucosal injury in this entity.

MATERIALS AND METHODS

Animals

Our studies used the homozygous MMP-9-deficient male mice with FVB background and wild-type (wt) purchased from Jackson Laboratories (Ca, USA). The animals were 8 wk to 10 wk old and weighted from 25 g to 30 g. They were maintained in a restricted-access room with controlled temperature (23°C) and light-dark cycle (12 h:12 h) and were housed in rack-mounted cages with a maximum of 10 mice per cage. Mice were allowed to drink and feed *ad libitum*. The study was approved by the local Animal Welfare Committee and conformed to the principles of laboratory animal care and use (NIH publication 86-23).

Genotyping analysis

Liver samples from selected mice were collected and genotype was verified by polymerase chain reaction (PCR) using a sense oligonucleotide primer (5'-GCATACTTGTTACCGCTATGG-3') and an antisense oligonucleotide primer (5'-TAACCGGAGGTCCAAACTGG-3'). For the neomycin cassette, we also used a sense oligonucleotide primer (5'-GAAGGGACTGGCTGCTATTG-3') and an antisense primer (5'-AATATCACGGGTAGCCAACG-3'). All procedures were performed blind with respect to genotype.

Experimental design

Distal colitis was induced by oral DSS (molecular wt 40000; ICN Biomedicals, Aurora, OH) at 5% in tap water *ad libitum* for 5 d in wt and MMP-9 deficient mice and then switched to water and monitored for 7 additional days. At least 15 age and sex matched wt and MMP-9 deficient mice were studied at the end of 5 d with DSS treatment and at 7 d after DSS (recovery phase), whereas 15 control wt and 15 control MMP-9 deficient mice were allowed free access to drinking water. Body weight was routinely obtained every second day and mortality was recorded at any time. Mice were euthanized by cervical dislocation on d 5 and 12 after induction of colitis. With the use of sterile equipment, a mid laparotomy was performed, the colon was removed, opened longitudinally, rinsed with sterile saline, and divided into two parts by a longitudinal section. One specimen was homogenized and stored at -20°C for MMPs, TIMPs, TNF- α and myeloperoxidase (MPO) assay. The second specimen was used for microscopic assessment of mucosal lesions.

Isolation of neutrophils

Mice were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg, Park Davis, Morris Plains, NJ). Blood samples were collected from the abdominal aorta. Neutrophils were isolated using Histopaque (Sigma Ltd, England) and erythrocyte removed using hypotonic lysis with ammonium chloride. The neutrophil count was determined by using a Neubauer hemocytometer and cell viability was assessed by trypan blue exclusion. Typically, the neutrophil preparations were > 98% pure and > 94% viable. The isolated neutrophils were washed and resuspended in HBSS with calcium and magnesium at a concentration of 5×10^9 cells/L and incubated for 12 h at 37°C. The release of MMPs was determined in the supernatant by zymography^[19].

Cell culture

Caco-2 cells, a human intestinal epithelial cell line, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in 75-cm² flasks in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were grown in minimum essential medium supplemented with pyruvate, sodium bicarbonate, 20% fetal bovine serum, gentamycin sulfate (0.05 mg/mL), penicillin G (0.06 mg/mL) and streptomycin sulfate (0.01 mg/mL). The medium was changed every two days and cells were subcultured three times each week. When confluent, cells were detached using a Trypsin-EDTA solution. For the *in vitro* experiments, Caco-2 cells were seeded into 24-well plates (30 000 cells per well) in free serum-medium and cultured in the presence or absence of human recombinant TNF- α (1 or 10 μ g/L) for 24 h. Afterwards, conditioned media was collected and stored at -20°C for the zymography assay and Western blot analysis.

Analytical Methods

Zymography: The activity of pro-MMP-9 was measured as previously described^[20,21] in homogenates of colonic

tissue, supernatants of purified neutrophils and Caco-2 conditioned media. Briefly, samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with copolymerized gelatin (0.2%; Sigma Chemical Co, St. Louis, Mo). After electrophoresis, the gels were washed with 2% Triton X-100 (2 times, 20 min each), and then incubated in development buffer (50 mmol/L Tris HCl, 200 mmol/L NaCl, 10 mmol/L CaCl₂ and 1 μ mol/L ZnCl₂, pH = 7.5) at 37°C overnight. Human recombinant MMP-9 and MMP-2 (Oncogene Research, Nottingham, UK), conditioned medium of HT-1080 human fibrosarcoma cells and molecular weight markers were used as standards. After incubation, gels were fixed and stained in 40% methanol, 10% acetic acid and 0.1% (wt/v) Coomassie Blue for 1 h and then de-stained. The low availability of active MMP-9 seen in the zymographies may be due to its high level of instability and the removal of active enzyme during the washing of specimens, as it has been previously suggested^[22].

Reverse zymography: The TIMP-1 activity was analyzed in homogenates of colonic tissues, as previously described^[23]. Briefly, samples were subjected to 15% SDS-PAGE with copolymerized 0.2% gelatin and human recombinant MMP-2 (160 μ g/L). Human recombinant TIMP-1 was used as the internal standard. TIMP-1 activity was identified by inhibition of gelatinolysis when compared with the standard.

Western-blot: Western-blot analysis of MMP-9 was performed in homogenates of colonic tissue and Caco-2 conditioned media, as previously described^[20]. Samples were denatured and loaded (50 μ g/lane) onto 10% (vol/vol) polyacrylamide gels. Following electrophoresis, proteins were transferred onto a nitrocellulose membrane (Protran, Schleider&Schuell) and detected using a rabbit monoclonal anti-MMP-9 antibody (Chemicon, Ca, USA) at 1:1000 concentration and a chemiluminescent substrate (Pierce, Rockford, USA). To account for the inter-blot variations in MMP immunoreactivity, an internal standard (conditioned medium of HT-1080 cells, and recombinant MMP-9) was used^[18]. Western Blotting with monoclonal anti-beta-actin (Sigma Chemical Co, St. Louis, Mo) was performed as an internal control.

Bands quantification: Clear bands were analyzed using a calibrated densitometer (GS-800, BioRad) and Quantity One Quantitation analysis software (BioRad, version 4). Each band was measured in terms of Optical Density Units of trace quantity (ODu) \times mm.

Quantification of TNF- α by ELISA: TNF- α expression was assayed in colonic tissues. Fragments of colon (40-50 mg) from the proximal region of the rectum were collected from wt and KO animals treated as described above. Afterwards, colon tissues were placed in 500 mL ice-cold PBS containing 20 μ L of a proteinase inhibitor cocktail (Roche Diagnostic GmbH, San Francisco, CA) and immediately sonicated. After 10 min in ice, cellular debris was removed by centrifugation at 10000 g for 10 min at 4°C and the protein content of the supernatant was assayed by Bradford's method. The specific ELISAs for mouse TNF- α used in these experiments were purchased from BD Biosciences (San Diego, CA). Murine TNF- α concen-

Table 1 Microscopic assessment of histologic changes in DSS colitis

A: Grade of crypt lesion	Score
Intact crypt	0
Grade 1: Loss of the basal third	1
Grade 2: Loss of two thirds	2
Grade 3: Loss of entire crypt	3
Grade 4: Erosion	4
B: Extension of each grade of crypt lesion as % of total mucosal surface	0-100
Crypt lesion score	Σ (A \times B)
Grade of inflammation, epithelial regeneration or crypt distortion	Score
Absent	0
Mild	1
Moderate	2
Severe	3

Crypt lesion, acute and chronic inflammation, epithelial regeneration and crypt distortion are scored separately.

trations in tissue extracts were assayed in duplicate wells following the manufacturer's recommendations. The sensitivity of ELISA for TNF- α in our laboratory was 20 ng/L. Data were expressed as pg TNF- α / μ g of total protein.

MPO assay: For the MPO activity assay^[24], the colonic specimen was homogenized in 2 mL phosphate-buffered saline, using a Tissue Tearor (model 985-370, Biospec, Racine, WI), and centrifuged. The pellets were again homogenized in an equivalent volume of phosphate buffer (50 mmol/L, pH 6) containing 0.5% hexadecyltrimethylammonium bromide (Sigma) and 5 mmol/L EDTA, sonicated three times for 30 s each time (Labasonic 2000, Braun), and centrifuged. Supernatants were used for determination of tissue MPO activity by a kinetic method. One unit of enzyme activity is defined as the amount of MPO that degrades 1 mmol of peroxide per minute at 25°C.

Histological Measurement of Colonic Lesions

Colonic specimens were fixed in formalin and coded for blind microscopic measurement of mucosal lesions. Samples were embedded in paraffin using a "Swiss roll" technique, and longitudinal sections from cecum to rectum were prepared and stained with hematoxylin and eosin. This procedure allows the examination of the entire specimen from cecum to rectum in every section. Two pathologists who were unaware of the treatment measured the extent of mucosal surface involved by crypt lesions as a percentage of the total colonic surface. For each specimen, a crypt damage score was obtained by summation of the products of each grade of crypt damage times the extent of mucosal surface involved by this grade of damage (Table 1). In addition, the specimens were graded 0 to 3 for inflammation (acute + chronic), regenerative changes (hyperplastic epithelium) and crypt distortion (distorted epithelium) using the criteria described by Cooper *et al*^[25]. Acute inflammation scores evaluated of the presence of polymorphonuclear neutrophils in the infiltrate, whereas chronic inflammation scores evaluate the presence of mononuclear cells.

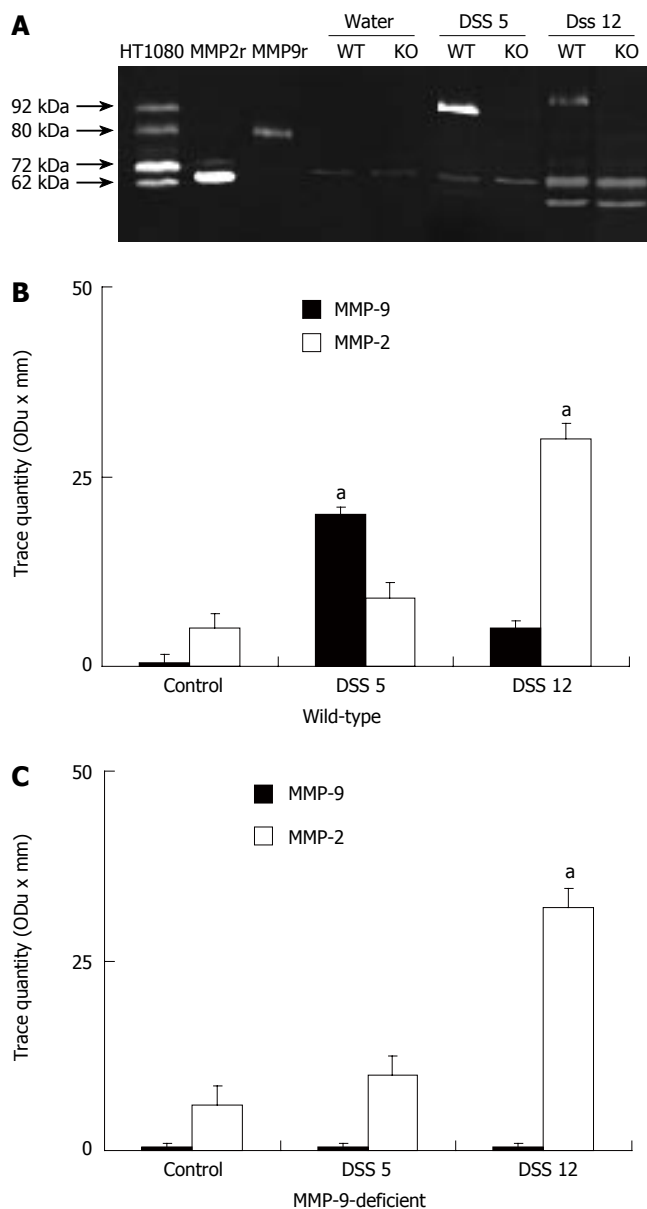


Figure 1 Gelatinase activity in colonic homogenates. **A:** Representative zymogram in wild-type (wt) and MMP-9-deficient (KO) animals on d 5 (DSS 5) and 12 (DSS 12) after induction of colitis ($n = 9$ in each group). Supernatant of HT1080 cells, human recombinants MMP-9 (rMMP9) and MMP-2 (rMMP2) were used as positive controls. Gelatinases with molecular weights of 62, 72 and 92 kDa corresponding to activated MMP-2, pro-MMP-2 and pro-MMP-9, respectively, were detected. Quantitative data of pro-MMP-9 and total MMP-2 (pro-MMP-2 and MMP-2) ($^aP < 0.05$ vs control) in wt (**B**) and MMP-9-deficient mice (**C**).

Statistical analysis

Results are presented as mean \pm SE. The statistical difference between means was determined using one way analysis of variance for overall comparison and the Student-Newman-Keuls test as post-test for single comparisons. The mortality data were analyzed by Kaplan-Meier survival curves.

RESULTS

DSS Colitis

Exposure to 5% DSS in drinking water for 5 d induced diarrhea and rectal bleeding in all mice. Furthermore, DSS treatment resulted in a significant increase in MPO activity

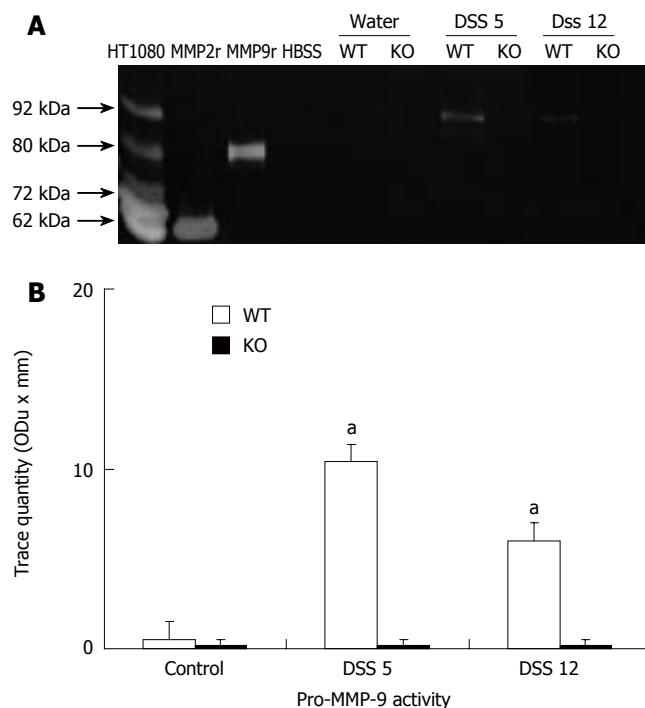


Figure 2 Gelatinase Activity in purified Neutrophils. **A:** Representative zymogram in wild-type (wt) and MMP-9-deficient (KO) animals on d 5 (DSS 5) and 12 (DSS 12) after induction of colitis ($n = 15$ in each group). Supernatant of HT1080 cells, human recombinants MMP-9 (rMMP9) and MMP-2 (rMMP2) were used as positive controls. HBSS was used as negative control. Pro-MMP-9 was detected in neutrophils from colitic wt animals; **B:** Quantitative data ($^aP < 0.05$ vs controls).

in the colonic tissues of both MMP-9 deficient mice (colitic: 2.5 ± 0.82 vs control: 0.3 ± 0.05 mU/mg protein, $P < 0.05$) and wt (colitic: 1.95 ± 0.67 vs control: 0.25 ± 0.06 mU/mg protein, $P < 0.05$). Histological examination of colonic sections revealed that administration of DSS resulted in epithelium injury and loss of normal crypt architecture, with some areas of erosion. Mixed infiltrate of neutrophils and mononuclear cells was observed in the lamina propria and submucosa. The muscularis propria was not involved. These changes were diffuse but predominated at distal parts of the colon. Histological examination revealed no changes in colons from normal control mice.

Gelatinase activity in DSS colitis

Zymographies revealed that MMP-2 but not pro-MMP-9, was a dominant gelatinase in colonic homogenates from non-colitic animals in both wt and deficient animals (Figure 1A). However, DSS treatment resulted in a significant up-regulation of pro-MMP-9, but not MMP-2 activity in colitic wt animals in comparison to deficient animals on d 5 after the induction of colitis (Figure 1A and B). On d 12, pro-MMP-9 activity was still up-regulated in wt animals, although to a less extent than in d 5. In addition, there was an up-regulation of MMP-2 in both groups (Figure 1A and B). In peripheral neutrophils isolated from wt animals pro-MMP-9 activity was also markedly enhanced on d 5 and, to a lesser extent, on d 12 following the induction of colitis, but not from MMP-9 deficient mice (Figure 2A and B). By contrast, no MMP-2 activity was shown in neutrophils isolated from any animal group in the presence or absence of DSS treatment.

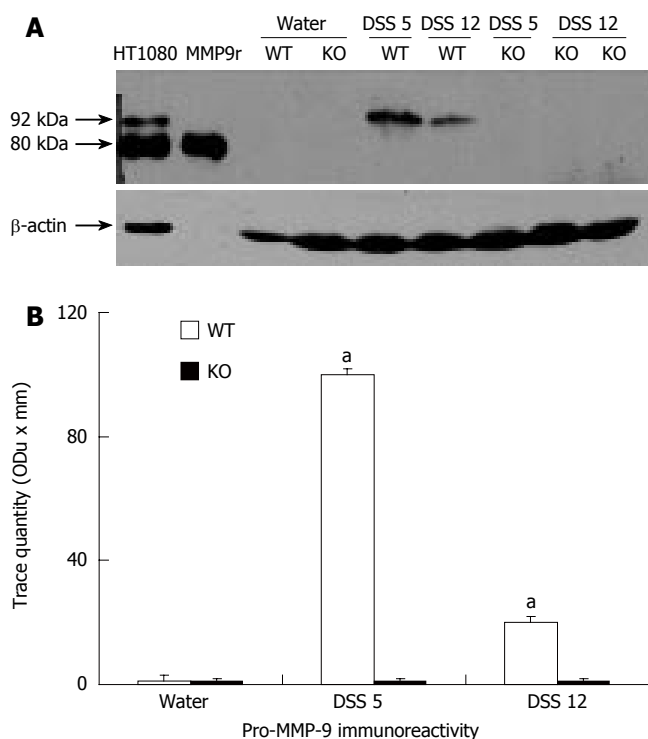


Figure 3 Pro-MMP-9 immunoreactivity in colonic homogenates. **A:** Representative immunoblot in wild-type (wt) and MMP-9-deficient (KO) ($n = 9$ in each group) animals on d 5 (DSS 5) and 12 (DSS 12) after colitis induction. Supernatant of HT1080 cells and human recombinants MMP-9 (rMMP9) were used as positive controls. A band of 92 kDa corresponding to pro-MMP-9 was detected in colitic samples. **B:** Quantitative analysis ($^aP < 0.05$ vs control).

DSS Colitis Enhanced MMP-9 Immunoreactivity

As shown by the immunoreactive band migrating at 92 kDa, a significant up-regulation of pro-MMP-9 was observed in wt, but not in controls or MMP-9 deficient mice, on d 5 and 12 following the induction of colitis (Figure 3).

TIMP-1 Activity in DSS Colitis

Colitis resulted in a significant up-regulation of TIMP-1 in wt animals receiving DSS compared to controls on d 5 and this increase remained elevated on d 12 after induction of colitis (Figure 4A and B). In wt mice the pro-MMP-9/TIMP-1 ratio was significantly higher ($P < 0.05$) than in control mice on d 5 and 12 after the induction of colitis. In addition, the pro-MMP-9/TIMP-1 ratio was not altered by colitis in MMP-9-deficient mice (Figure 4C).

TNF- α Content in DSS Colitis

Figure 5 shows that wt and MMP-9-deficient animals exposed to DSS for during 5 d showed a significant increase in TNF- α concentration as compared with non-colitic animals. In addition, the TNF- α level remained elevated in wt animals on d 12 after colitis induction, whereas returned to base line levels in MMP-9 deficient animals.

Effect of TNF- α Treatment

Since in DSS-induced colitis the predominant lesions are circumscribed to the mucosa, we measured the MMP activity in intestinal epithelial (Caco-2) cells in response to TNF- α . Zymographies showed that, under basal conditions, MMP-2 was the predominant gelatinase in Caco-2

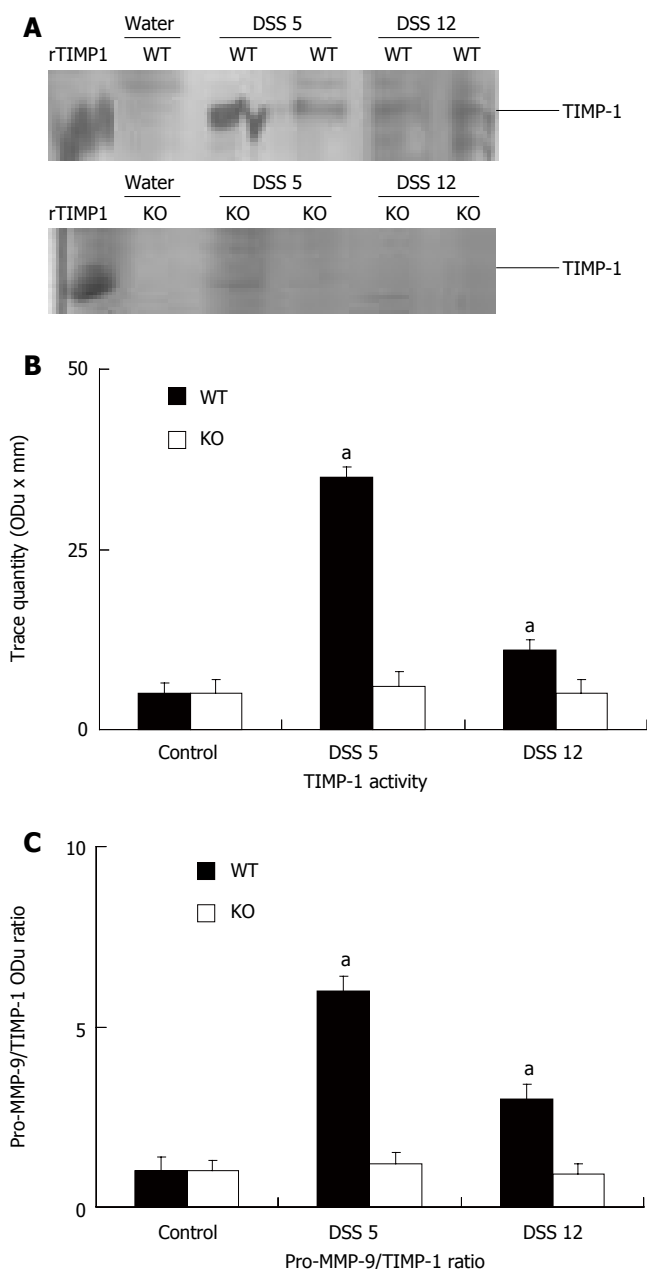


Figure 4 TIMP-1 activity in DSS-induced colitis: **A:** Representative reverse zymograms in homogenates of colonic tissue in wild-type (wt) and MMP-9-deficient (KO) animals on d 5 (DSS 5) and 12 (DSS 12) after induction of colitis; **B:** Quantitative data of TIMP-1 activity ($^aP < 0.05$ vs control) ($n = 5$ in each group); **C:** wt mice had a pro-MMP-9/TIMP-1 ratio significantly ($^aP < 0.05$) higher than controls on d 5 (DSS 5) and 12 (DSS 12) following the induction of colitis. The pro-MMP9/TIMP-1 ratio was not modified by DSS-induced colitis in KO mice.

cells. Interestingly, TNF- α treatment resulted in a dose-dependent significant increase of pro-MMP-9 activity whereas MMP-2 activity remained unchanged (Figure 6A and C). In addition, Western blot analysis corroborated that TNF- α treatment significantly increased pro-MMP-9 immunoreactivity in intestinal epithelial cells (Figure 6B and D).

Impact of MMP-9-silencing on DSS-colitis outcome

Both wt and MMP-9-deficient mice developed diarrhea and rectal bleeding with DSS. Percent loss of body weight is shown in Figure 7A. Neither deficient nor wt mice showed major loss of body weight during DSS treatment.

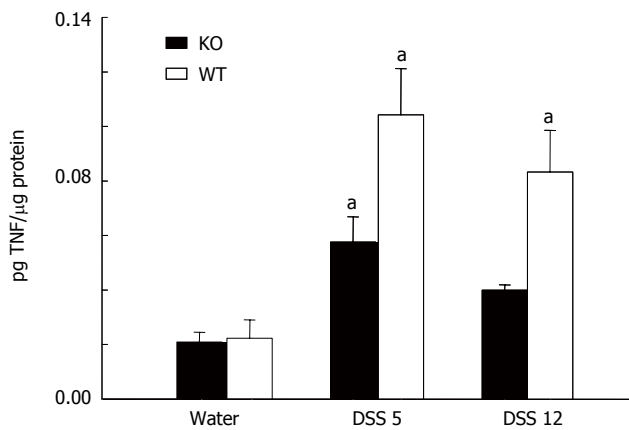


Figure 5 TNF- α content in colonic tissue. Concentration of TNF- α protein in homogenates of colonic tissue from wild-type (wt) ($n = 5$) and deficient (KO) ($n = 5$) animals on d 5 (DSS 5) and 12 (DSS 12) after colitis induction ($^aP < 0.05$ vs control).

However, during the recovery phase, wt mice showed significant weight loss. In addition, the mortality was significantly higher in wt than in MMP-9 KO mice (Figure 7B). The mean of severity of crypt damage, inflammation, epithelial regeneration and crypt distortion scores are summarized in Table 2. MMP-9 deficient mice showed a significant decrease in the crypt damage scores when compared with wt mice on d 5 and 12 after colitis induction. Moreover, deficient mice showed significantly lower scores of acute and chronic inflammation on d 12. Epithelial regeneration and crypt distortion scores were almost absent on d 5, and no differences were found between both groups at any time.

DISCUSSION

The etiology and the cascade of the events resulting in intestinal injury of UC patients still remain unclear. There is growing evidence that MMPs are implicated in tissue remodeling and destruction associated with several inflammatory conditions^[6-8], including IBD^[9-13]. Moreover, increased gelatinase activity has been shown in diverse animal experimental models of colitis^[17,26]. Our study revealed that MMP-9-deficient animals showed improved survival and indices of epithelial injury, suggesting that MMP-9 is a key factor in tissue damage in DSS-induced colitis.

The destructive effect of MMPs in the gut has been shown by several *in vitro* studies^[27-29]. In addition, previous reports have found increased levels of MMP-9 in colonic tissues of patients with IBD. For instance, Bailey *et al*^[30] have shown an increased MMP-9 protein in patients with IBD by immunohistochemical techniques. Furthermore, Baugh *et al* have found that MMP-9 activity measured by zymography was the most abundant MMP expressed in intestinal tissues of patients with UC compared with normal controls^[9]. Moreover, Tartlon *et al*^[26] have also shown increased gelatinase activity in a transmural experimental model of colitis induced in immunodeficient mice. Finally, we have previously found that MMP-9 is up-regulated in intestinal tissues from rats with DSS-induced

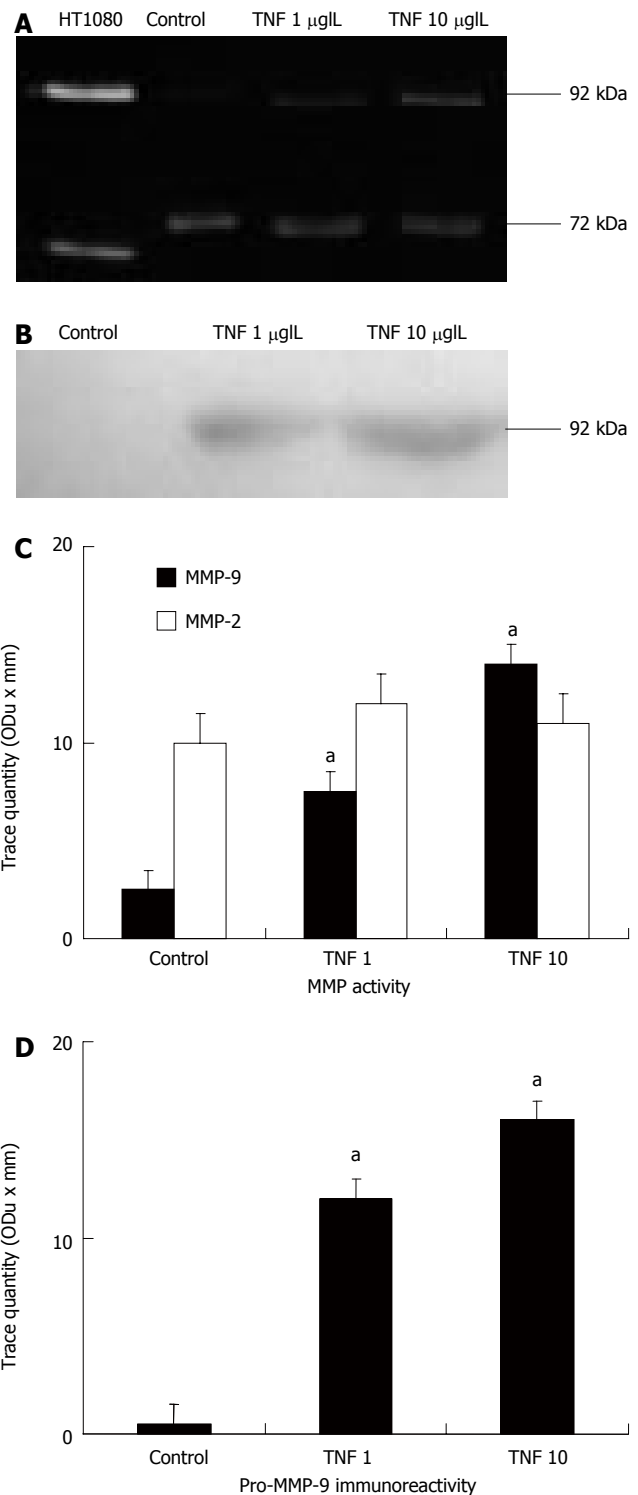


Figure 6 Activity of gelatinases in Caco-2 cells in absence (control) or presence of TNF- α (1 μ g/L or 10 μ g/L). **A**: Representative zymogram of gelatinases with molecular weights of 72 and 92 kDa corresponding to pro-MMP-2 and pro-MMP-9, respectively, were detected. Supernatants of HT1080 cells were used as controls. **B**: Representative immunoblot of pro-MMP-9 (92 kDa) protein in Caco-2 cells as above. **C**: Quantitative zymographic analysis ($^aP < 0.05$ vs control). **D**: Quantitative western-blot analysis ($^aP < 0.05$ vs control) ($n = 3$ in each group).

colitis^[17]. Our present study shows that DSS treatment induced an increased activity and expression of pro-MMP-9 in wt mice compared to the MMP-9 deficient group. It could be surprising that DSS did not induce the active form of MMP-9, however, the low availability

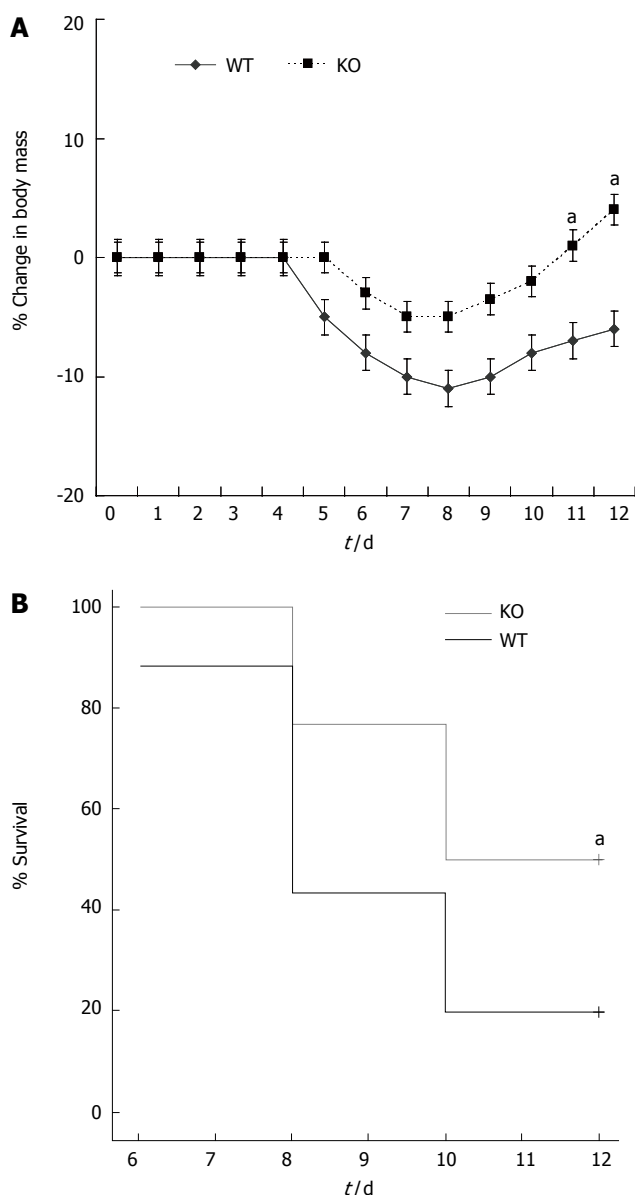


Figure 7 **A:** Percentage of body weight change during DSS-induced colitis in wild-type (WT) and deficient (KO) animals ($n = 30$ in each group). Values are mean \pm SE of percentage body weight change in each animal relative to weight at the start of DSS treatment. WT animals showed more weight loss than deficient mice ($^aP < 0.05$). **B:** Survival curve of both colitic groups. The mortality was significantly higher in wt animals compared to deficient mice ($^aP = 0.015$).

of this enzyme may be due to the removal of the active form of MMP-9 during the washing of specimens, as it has been previously suggested^[22]. Our data also show that colitis induced by DSS was enhanced in wt animals. In fact, histological evidence of less mucosal damage was observed in MMP-9 deficient animals on d 5 and 12 after induction of colitis. In addition, the higher weight loss and mortality of wt mice compared with deficient animals supports this hypothesis. In our experiments the indices of DSS-induced colitic injury correlated well with pro-MMP-9, but not with MMP-2 up-regulation. By contrast, whereas MMP-2 activity was elevated during the recovery phase of colitis, the activity and expression of pro-MMP-9 decreased on d 12 after colitis induction. These data suggest that MMP-9 is mainly implicated in mucosal injury at the early stage of colitis, whereas MMP-2 seems to be linked to mucosal

Table 2 Histologic scores on d 5 and 12 after Induction of colitis by DSS

	D 5		D 12	
	WT	KO	WT	KO
Crypt lesion	40.0 \pm 4.0	21.0 \pm 5.0 ^a	18.0 \pm 2.4	9.5 \pm 2.4 ^a
Acute inflammation	1.0 \pm 0.2	0.9 \pm 0.1	1.8 \pm 0.2	0.5 \pm 0.15 ^a
Chronic inflammation	0.5 \pm 0.1	0.6 \pm 0.1	1.5 \pm 0.10	0.4 \pm 0.1 ^a
Epithelial regeneration	0.2 \pm 0.05	0.1 \pm 0.02	1.2 \pm 0.15	0.95 \pm 0.2
Crypt distortion	0	0	0.6 \pm 0.10	0.5 \pm 0.2

^a $P < 0.05$ vs WT.

repair, although MMP-2 deficient animals should be tested to confirm this hypothesis. Therefore, further investigation is warranted.

In many inflammatory conditions, the excess degradation of ECM may result from an imbalance between MMP and TIMP activity^[31]. Since TIMP-1 seems to be the major inhibitor of gelatinase activity^[3] we studied the activity of this inhibitor in DSS-induced colitis. We found that TIMP-1 was increased in colitic wt animals on d 5 after induction of colitis in comparison with non-colitic mice. However, our results showed an imbalance between pro-MMP-9 and TIMP-1 activity in colitic wt animals in favour of MMP-9, suggesting that the increase of TIMP-1 was insufficient in preventing pro-MMP-9 from exerting its biologic effects in DSS-induced colitis. Interestingly, in MMP-9 deficient animals DSS treatment did not increase TIMP-1 activity, which could suggest an adapted regulatory response of this endogenous inhibitor to the levels of pro-MMP-9. Our results are consistent with previous studies in human IBD where an imbalance between MMPs and their endogenous inhibitors has been shown^[10,12].

We next studied the cellular origin of MMP-9 in DSS-induced colitis. MMP-9 released from neutrophils could be an important factor for transmigration and proteolysis of ECM in several inflammatory conditions contributing to tissue injury^[19,32]. We found that pro-MMP-9 is expressed by neutrophils only in colitic wt animals. However, DSS treatment did not induce any significant difference in MPO activity and inflammation score (markers of neutrophilic infiltration) between wt and MMP-9 deficient animals on d 5. This is consistent with the fact that inflammation is thought to be a secondary event that follows epithelial damage in this experimental model of colitis^[25]. Therefore, it seems reasonable that other cells may also generate MMP-9 contributing to crypt damage during the initial phase of DSS-induced colitis. Intestinal epithelium plays an important role in the immunomodulatory response of the intestinal mucosa^[33,34]. Indeed, the loss of intestinal epithelium integrity could lead to an interaction between the luminal antigenic stimuli with the mucosal immune system, resulting in intestinal inflammation^[35-37]. We used a human intestinal epithelial cell line to confirm whether MMP-9 and MMP-2 are up-regulated by inflammatory stimuli, as previously described^[38]. Consistent with the colonic tissue samples, we found that TNF- α treatment only resulted in up-regulation of pro-MMP-9 activity in intestinal epithelial cells. These data therefore suggest that MMP-9 could

be released from inflamed intestinal epithelial cells with the subsequent loss of mucosal integrity, thus facilitating the penetration of inflammatory cells such as PMN into inflamed tissue in the late inflammatory phase of DSS-induced colitis. Our results are consistent with a recent study where epithelial MMP-9 has been shown as an important factor for tissue damage using the Caco-2 intestinal cell line, since MMP-9 inhibited cell attachment and wound healing in an *in vitro* model^[39]. In addition, epithelial protection in MMP-9-deficient mice during the early phase of colitis could also account for a mitigated inflammatory response in the late phase of this experimental model. In fact, only on d 12 after DSS administration MMP-9-deficient animals showed lower inflammation scores than wt animals. These results are consistent with our previous study, where MMP inhibition with CGS-27023-A treatment in rats with DSS-induced colitis significantly reduced the inflammation scores only during the recovery phase of the colitis^[17].

In conclusion, this study presents direct evidence that DSS-induced colitis is attenuated in mice deficient in MMP-9. The reduction in inflammatory indices and pathological scoring suggests a link between MMP-9 and DSS-induced colitis that might help elucidate the physiopathology of UC. These results suggest that selective inhibition of this gelatinase could be a novel therapeutic strategy for patients with UC.

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BASIC RESEARCH

Gene expression profiles of hepatic cell-type specific marker genes in progression of liver fibrosis

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genesis. Sequential activation of inflammatory cells and the self-supporting properties of HSCs play an important role in development of fibrosis.

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Key words: Liver fibrosis; Gene expression; Microarray; Dimethylnitrosamine; Marker genes; Hepatic stellate cell; Kupffer cell; Hepatocytes; Metabolic pathway

Takahara Y, Takahashi M, Wagatsuma H, Yokoya F, Zhang QW, Yamaguchi M, Aburatani H, Kawada N. Gene expression profiles of hepatic cell-type specific marker genes in progression of liver fibrosis. *World J Gastroenterol* 2006; 12(40): 6473-6499

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Abstract

AIM: To determine the gene expression profile data for the whole liver during development of dimethylnitrosamine (DMN)-induced hepatic fibrosis.

METHODS: Marker genes were identified for different types of hepatic cells, including hepatic stellate cells (HSCs), Kupffer cells (including other inflammatory cells), and hepatocytes, using independent temporal DNA microarray data obtained from isolated hepatic cells.

RESULTS: The cell-type analysis of gene expression gave several key results and led to formation of three hypotheses: (1) changes in the expression of HSC-specific marker genes during fibrosis were similar to gene expression data in *in vitro* cultured HSCs, suggesting a major role of the self-activating characteristics of HSCs in formation of fibrosis; (2) expression of mast cell-specific marker genes reached a peak during liver fibrosis, suggesting a possible role of mast cells in formation of fibrosis; and (3) abnormal expression of hepatocyte-specific marker genes was found across several metabolic pathways during fibrosis, including sulfur-containing amino acid metabolism, fatty acid metabolism, and drug metabolism, suggesting a mechanistic relationship between these abnormalities and symptoms of liver fibrosis.

CONCLUSION: Analysis of marker genes for specific hepatic cell types can identify the key aspects of fibro-

INTRODUCTION

The pathological relationship between chronic inflammation and formation of fibrosis has been established in various organs, including the liver, kidney, lung and pancreas. Although liver fibrosis has been studied extensively, the underlying mechanisms remain unclear and drugs to prevent and treat fibrosis are only partially effective. DNA microarray technology offers an approach to this kind of complex problems, and microarray analyses of the whole liver have been reported for liver fibrosis^[1,2]. However, these analyses did not address the behavior of individual hepatic cell-types and the interactions of hepatic cells during fibrosis. Therefore, in the current study we identified hepatic cell-specific marker genes that could be used to understand the *in vivo* behavior of each type of hepatic cells during fibrogenesis.

About 70%-80% of hepatic cells are parenchymal hepatocytes, while the non-parenchymal cells are mainly composed of Kupffer cells, hepatic stellate cells (HSC) and sinusoidal endothelial cells (SECs)^[3,4]. Kupffer cells are the resident monocytes in liver, and act in phagocytosis of foreign substances such as microorganisms, as well as management of inflammatory processes. Kupffer cells and infiltrated monocytes and lymphocytes are considered to trigger inflammation in the early phase of hepatitis and then maintain chronic inflammation. HSCs control hepatic and cardiovascular contraction, and produce extracellular

matrix (ECM) components and cytokines for repair of organs. HSCs are also believed to have a central role in hepatic fibrosis formation. Hepatocytes fulfill the main functions of the liver, including regulation of nutrition, production of major serum proteins, and elimination of unnecessary materials to maintain homeostasis of the whole body^[4].

In the current study, we first examined gene expression profiles of hepatic cells that were isolated at different time points during liver fibrosis. Marker genes specific for different types of hepatic cells were obtained by comparing these profiles and using information from previous studies. DNA microarray data for the whole liver were then interpreted during liver fibrogenesis using the hepatic cell-type specific marker genes. Our results suggest that new pathological properties and intracellular events are associated with each cell type during liver fibrogenesis, and provide candidates for diagnostic markers of liver fibrosis.

MATERIALS AND METHODS

Animals and experimental protocols

Male Sprague-Dawley rats (Charles River Japan, Yokohama, Japan) weighing 160-190 g were housed with unrestricted access to food (CRF-1, Oriental Yeast, Tokyo, Japan) and water in air-conditioned animal quarters with a 12 h light/dark cycle (light between 07:00 and 19:00 h). Hepatic fibrosis was induced by intraperitoneal injection of 0.5% dimethylnitrosamine (DMN; Wako Pure Chemical Industries, Osaka, Japan) at 2 mL/kg of body weight for three consecutive days each week for four weeks. Blood samples were drawn from the inferior vena cava on d 0, 4, 7, 14, 21 and 28, respectively. Liver specimens obtained on these days were dissected and immediately frozen in liquid nitrogen. Fibrosis was confirmed by haematoxylin and eosin (HE) staining of the liver tissue. The hydroxyproline content of liver specimens was determined as previously described by Horie *et al*^[5]. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using commercial kits (Fuji Film, Tokyo, Japan), and hyaluronic acid levels were determined using a commercial ELISA kit (Fujirebio, Tokyo, Japan). The animal facilities and protocol were reviewed and approved by the Institutional Animal Care and Use Committee of Ajinomoto Co., Inc.

Preparation of HSCs, inflammatory cells (including Kupffer cells) and hepatocytes

HSCs were isolated from rat liver using the pronase-collagenase digestion method as previously reported^[6]. Kupffer cell fraction was prepared with an elutriator, using essentially the same method as previously described^[6], and hepatocytes were isolated as previously described^[7].

Selection of marker genes for hepatic cells

Hepatic cells (HSCs, Kupffer cell fraction and hepatocytes) were isolated from the liver on d 0, 4, 7, 14, 21 and 28 during liver fibrogenesis. The isolated hepatic cells at each time point were subjected to DNA microarray analysis. If

the maximum or minimum expression of a gene in hepatocytes was ten times higher or lower than that of the same gene in HSCs and the Kupffer cell fraction, the up- or down-regulated gene was defined as a hepatocyte-specific marker gene. If the maximum or minimum expression of a gene in HSCs was ten times higher or lower than that of the same gene in hepatocytes and three times higher or lower than the same gene in the Kupffer cell fraction, this gene was defined as a HSC-specific marker gene. Similarly, if the maximum or minimum expression of a gene in the Kupffer cell fraction was ten times higher or lower than that of the same gene in hepatocytes and three times higher or lower than the same gene in HSCs, the gene was defined as an inflammatory cell-specific marker gene. An explanation of the use of the term 'inflammatory cell', rather than 'Kupffer cell', was given in the Results section. The ratios used to determine cellular specificity were based on previous reports^[8,9]: the number of hepatocytes was about ten times higher than that of the inflammatory cell fraction or HSCs, while the number of Kupffer cells was similar to that of HSCs. Use of a high ratio improved the definition of cellular specificity, but also decreased the number of marker genes. However, we found that these ratios were the most appropriate for identification of a set of genes for analysis of liver fibrogenesis.

Microarray analysis

RNAs both from frozen liver tissues and from each isolated cell type were prepared using Isogen reagent (Nippon Gene), and the quality and quantity of each RNA sample were assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.). RNA samples were reverse-transcribed with a poly (dT) oligonucleotide attached to a T7 promoter and copied into dsDNA (Invitrogen). *In vitro* RNA transcription was performed to incorporate biotin-labeled ribonucleotides into the cRNA transcripts using a RNA transcript labeling kit (Enzo Biochem). Some of the RNA (15 g) was utilized for hybridization to a rat genome U34A array (Affymetrix) and a quality assay using test 3 array probe chips was performed according to the manufacturer's protocol. After hybridization and subsequent washing using the Affymetrix Fluidics Station 400, fluorescence signals amplified with streptavidin phycoerythrin were measured using the Affymetrix scanner, and the results were analyzed using the MicroArray suite software. In the whole liver analysis, two rats and two arrays were used at each time point (d 4, 7, 14, 21 and 28). In the cell-type-specific analysis, one rat which was selected by ALT/AST score and one array were used at each time point (d 4, 7, 14, 21 and 28).

Statistical analysis

Clustering of K-means was performed using the TIGR MeV (MultiExperiment Viewer)^[10]. Gene expression profiles in the chronic phase were clustered into 10 patterns using K-means analysis. Clustered genes with a tendency to temporally decrease (clusters 1, 3 and 10) or increase (clusters 7 and 9) were selected as gene markers that had a strong relationship with fibrogenesis.

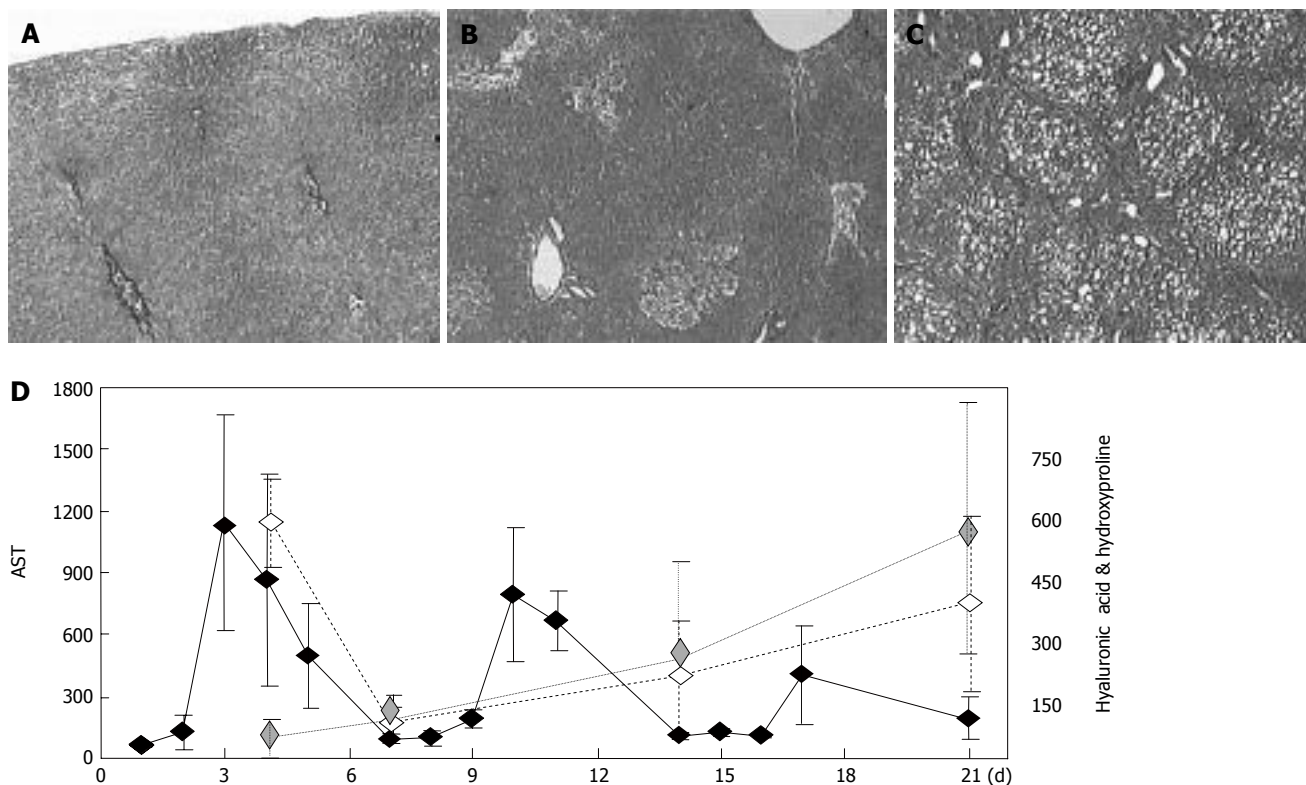


Figure 1 Histological and biochemical analyses of fibrogenesis. **A-C:** Histological staining (HE staining) of control liver sections (**A**) and sections obtained on d 4 (**B**) and d 21 (**C**), respectively, after DMN administration; **D:** Biochemical analysis of fibrogenesis showing plasma AST levels (IU/L, solid line), plasma hyaluronic acid levels (ng/mL, dashed line), and liver hydroxyproline levels (ng/mL, dotted line). The x-axis shows the days of fibrogenesis, and each value on the graph is shown as the mean \pm SE, $n = 5$.

RESULTS

Time course of gene expression profiles for the whole liver during fibrogenesis

Administration of DMN for three days induced an inflammatory reaction in liver cells simulating the active phase in hepatitis, and the subsequent lack of administration of DMN for four days was used to simulate the remission phase in hepatitis. Repetition of this cycle led to fibrosis in three or four weeks, as shown in Figure 1. AST (GOT) and ALT (GPT) increased on the days of DMN administration and decreased on the days during which DMN was not administered (Figure 1), but both AST and the hyaluronic acid content in serum, a marker of fibrosis^[11], gradually increased on days without DMN administration. The inflammatory reaction in periods without DMN administration was weaker than that in periods with DMN-administration, but gradually increased in intensity and response. Based on the behavior shown in Figure 1, gene expression profiles on d 4 (just after a period of DMN administration) were defined as representative of the acute phase response, and those on d 7, 14, 21 and 28 (just before a period of DMN administration) were defined as representative of the chronic phase response.

Marker genes indicating fibrotic activity of HSCs

HSCs were isolated from liver at each time point over the time course of development of DMN-induced fibrosis. Marker genes expressed mainly in HSCs were selected from a DNA microarray analysis, as described in the Materials and Methods, and the selected HSC-specific marker

genes are listed in Figure 2. In addition, HSCs isolated from normal rats were cultured *in vitro* for 7 d and DNA microarray analysis of these cells was performed on d 0, 4, and 7, respectively. Marker genes identified in isolated HSCs in the DMN-induced fibrosis model showed the same behavior in the *in vitro* culture, supporting the HSC specificity of the selected marker genes. The behavior of HSC-specific marker genes *in vivo* during fibrogenesis was analyzed using DNA microarray data for the whole liver at each time point during development of DMN-induced fibrosis. These data could indicate the actual behavior of the marker genes *in vivo*, since the data from isolated HSCs might contain some bias due to isolation stimuli. The behavior of HSC-specific marker genes is shown in Figure 2, and supplemental background data are provided in Figure 3. The genes were separated into 2 groups as shown in Figure 2. Group 1 contained marker genes that were linearly up-regulated during fibrogenesis and in the acute inflammation phase, whereas group 2 contained marker genes that were linearly up-regulated during fibrogenesis but not in the acute inflammation phase. Both groups could be further separated into 2 subgroups. Group 1-1 included marker genes that were linearly up-regulated during fibrogenesis and remained in an up-regulated state on d 28, and group 1-2 included marker genes that were also linearly up-regulated during fibrogenesis but then decreased in expression on d 28. Group 2 was similarly separated into groups 2-1 and 2-2. In summary, two groups of HSC-specific marker genes were identified, one in which the genes responded to inflammatory stimuli and the other in which the genes did not respond to such stimuli. Furthermore,

Table 1 Marker genes for hepatic stellate cells (HSCs)

Group	Probe ID	Annotation	Symbol	GenBank	Whole liver						<i>In vitro</i> cultured HSC		
					d 0	4	7	14	21	28	d 0	3	7
1	M24067_at	Serine proteinase inhibitor clade E member 1/plasminogen activator inhibitor-1 (PAI-1)	PAI1	M24067	10.6	9.3	1.6	2.1	1.8	2.1	6.0	29.5	14.8
	M23566exon_s_at	alpha-2-macroglobulin	A2M	M23566	191.8	2.3	0.6	1.1	1.0	2.3	182.9	2.8	0.8
	M55534mRNA_s_at	Crystallin alpha polypeptide 2/alpha-crystallin B chain	CRYAB	M55534	13.1	8.9	1.1	1.9	3.2	3.8	369.6	3.0	1.5
	Z12298cds_s_at	Decorin	Dcn	Z12298	17.9	2.8	2.5	2.9	3.8	5.6	470.1	2.5	2.8
	rc_AA891527_at	Four and a half LIM domains 2	Fhl2	AA891527	12.7	3.5	2.7	3.8	5.8	7.9	396.3	2.8	2.6
	S57478cds_s_at	Annexin A1/lipocortin I	Anxa1	S57478	14.4	2.6	1.7	1.9	1.8	2.3	188.3	3.4	2.5
	D50093_s_at	Prion protein (RaPrP gene for prion protein)	Prnp	D50093	2.2	46.0	7.3	19.1	31.4	37.3	489.8	2.1	1.8
	rc_AI231472_s_at	Collagen, type 1, alpha 1	Col1a1	AI231472	113.0	1.7	1.0	2.7	3.1	4.2	153.1	3.2	5.1
	rc_AA900769_s_at	Vascular alpha-actin/actin, alpha-2, smooth muscle, aorta	ACTA2	AA900769	24.0	8.5	1.8	10.1	15.4	22.2	56.4	12.1	12.5
	L00382cds_at	Skeletal muscle beta-tropomyosin and fibroblast tropomyosin 1, alternative/Tropomyosin 2	TPM2	L00382	3.4	3.2	2.0	4.1	5.4	7.4	1.6	69.0	62.3
	U57362_at	Procollagen, type XII, alpha 1 (collagen XII alpha 1)	Col12a1	U57362	6.3	1.8	2.0	1.9	2.6	2.8	47.1	3.1	2.8
	M14656_at	Secreted phosphoprotein 1/Sialoprotein	Spp1	M14656	7.4	17.8	2.0	2.1	2.4	20.8	28.7	34.2	31.9
	rc_AI172064_at	Lectin, galactose binding, soluble 1	Lgals1	AI172064	61.6	2.3	1.2	2.6	4.3	3.0	224.3	4.9	3.8
	rc_AA894345_at	Phosphoprotein enriched in astrocytes 15 (predicted)	"Pea15_predicted"	AA894345	20.5	2.2	1.2	2.0	2.3	2.1	112.0	2.1	1.8
	M83107_g_at	Transgelin (SM22-alpha)	Tagln	M83107	29.3	3.1	1.6	3.4	5.1	4.6	81.7	14.7	10.0
2	L03294_g_at	Lipoprotein lipase	Lpl	L03294	22.5	1.2	1.6	1.5	2.3	4.5	76.9	3.5	7.8
	rc_AI012030_at	Matrix Gla protein	Mgp	AI012030	120.4	1.0	0.9	1.5	2.6	5.3	236.2	2.5	4.3
	M80829_at	Troponin T2, cardiac	Tnnt2	M80829	15.7	1.2	0.7	1.1	1.8	2.6	14.9	18.4	15.5
	M22400_at	Glypican 3 /developmentally regulated intestinal protein (OCI-5)	GPC3	M22400	3.7	0.9	2.4	3.9	7.4	10.0	8.6	0.6	1.0
	D00680_at	Glutathione peroxidase 3/plasma glutathione peroxidase precursor	Gpx3	D00680	20.5	1.4	1.5	2.7	3.4	3.9	104.3	5.1	4.3
	rc_AA800844_s_at	Similar to Loxl protein /Loxl1=lysyl oxidase-like 1	LoxL1	AA800844	29.3	1.5	1.5	4.8	7.5	8.5	166.0	3.5	5.3
	S77494_s_at	Lysyl oxidase	Lox	S77494	27.5	0.8	0.8	1.9	2.4	3.0	26.3	29.1	38.0
	AF030358_g_at	Small inducible cytokine subfamily D, number1/chemokine CX3C motif, ligand 1	CX3CL1	AF030358	4.6	1.0	2.2	2.3	3.0	4.0	24.0	0.6	1.5
	X84039_at	Lumican	Lum	X84039	8.1	1.0	2.1	2.1	2.3	1.1	52.8	2.3	3.2
	U09540_g_at	Cytochrome P450, family 1, subfamily b, polypeptide 1	Cyp1b1	U09540	8.1	1.0	1.3	1.2	1.9	1.6	29.9	5.0	6.2

Expression profiles of the whole liver and *in vitro* cultured isolated HSCs were obtained using a rat genome U34A array (Affymetrix). Gene markers for HSCs are listed. For analysis of HSCs in the whole liver, expression intensities are given for d 0, and expression intensity data for d 4, 7, 14, 21 and 28 are shown as ratios to the d 0 expression data. The classification of group1 (2) corresponds to the presence (absence) of up-regulated peak in the acute inflammation phase. For analysis of *in vitro* cultured HSCs, expression levels for d 0 are also shown, and data for d 4 and 7 are similarly shown as ratios to the expression level on d 0. Italicized values indicate an "absent" call by the Affymetrix software. Bold text indicates the highest ratio in the chronic phase (d 7, 14, 21 and 28). □ between 0.667 and 1.5; ■ ≥ 1.5; ■ ≤ 0.667.

HSC-specific marker genes were found that could identify biological changes in HSCs in the late phase of fibrosis.

Marker genes indicating inflammatory activity in immune cell populations

The Kupffer cell fraction was separated from other hepatic cells at each time point during the course of fibrosis development, and marker genes expressed mainly in the Kupffer cell fraction were selected from the DNA microarray analysis. These marker genes are shown in Figure 4, and supplemental background data are provided in Figure

5. Marker genes in the Kupffer cell fraction indicated the presence of other hematopoietic cells, such as mast cells, lymphocytes, erythrocytes and their progenitors in this fraction, as shown in Figures 4 and 5. Since the source cell population for these marker genes could not be confirmed, a particular hematopoietic cell was postulated to be the source of each marker gene based on previous reports as shown in supplemental Table 1. These data indicated that the method used for isolation of Kupffer cells was not appropriate in fibrotic liver, although it was effective for isolation of normal liver cells. However, despite this

Table 2 Marker genes in hepatic stellate cells (HSC)

Group	Probe ID	Annotation	Symbol	Gen Bank	Whole liver							Isolated HSC							In vitro cultured HSC		
					d 0	4	7	14	21	28		d 0	4	7	14	21	28		d 0	3	7
Group 1	M24067	Serine proteinase inhibitor clade E member 1/plasminogen activator inhibitor-1 (PAI-1)	PAI1	M24067	10.6	9.3	1.6	2.1	1.8	2.1		565.8	2.6	1.4	2.5	2.2	1.8		6.0	29.5	14.8
	M23566	alpha-2-macroglobulin	A2M	M23566	191.8	2.3	0.6	1.1	1.0	2.3		34.3	0.5	4.1	7.9	9.4	47.0		182.9	2.8	0.8
	M55534	Crystallin alpha polypeptide 2/alpha-crystallin B chain	CRYAB	M55534	13.1	8.9	1.1	1.9	3.2	3.8		619.5	1.9	1.0	1.0	1.5	1.2		369.6	3.0	1.5
	Z12298cds_s_at	Decorin	Dcn	Z12298	17.9	2.8	2.5	2.9	3.8	5.6		384.5	2.5	1.6	2.3	2.4	3.5		470.1	2.5	2.8
	rc_AA891527_at	Four and a half LIM domains 2	Fhl2	AA891527	12.7	3.5	2.7	3.8	5.8	7.9		260.8	2.9	1.5	1.3	2.0	3.7		396.3	2.8	2.6
	S57478cds_s_at	Annexin A1/lipocortin I	Anxa1	S57478	14.4	2.6	1.7	1.9	1.8	2.3		464.2	1.6	0.9	1.3	1.8	2.5		188.3	3.4	2.5
	D50093	Prion protein (RaPrP gene for prion protein)	Prnp	D50093	2.2	46.0	7.3	19.1	31.4	37.3		361.6	4.3	0.9	1.0	2.9	3.7		489.8	2.1	1.8
	rc_AI231472_s_at	Collagen, type 1, alpha 1	Col1a1	AI231472	113.0	1.7	1.0	2.7	3.1	4.2		890.1	0.5	0.8	2.5	2.9	3.8		153.1	3.2	5.1
	rc_AA900769_s_at	Vascular alpha-actin/actin, alpha-2, smooth muscle, aorta	ACTA2	AA900769	24.0	8.5	1.8	10.1	15.4	22.2		68.0	21.8	18.0	10.8	20.3	15.2		56.4	12.1	12.5
	L00382cds_at	Skeletal muscle beta-tropomyosin and fibroblast tropomyosin 1, alternative/Tropomyosin 2	TPM2	L00382	3.4	3.2	2.0	4.1	5.4	7.4		34.9	2.9	2.3	1.6	6.0	10.2		1.6	69.0	62.3
	U57362_at	Procollagen, type XII, alpha 1 (collagen XII alpha 1)	Col12a1	U57326	6.3	1.8	2.0	1.9	2.6	2.8		73.4	2.2	1.1	1.2	2.0	2.9		47.1	3.1	2.8
	M14656_at	Secreted phosphoprotein 1/Sialoprotein	Spp1	M14656	7.4	17.8	2.0	2.1	2.4	20.8		8.5	25.1	12.1	11.1	18.6	204.3		28.7	34.2	31.9
	rc_AI172064_at	Lectin, galactose binding, soluble 1	Lgals1	AI172064	61.6	2.3	1.2	2.6	4.3	3.0		980.7	2.0	1.3	1.6	1.9	1.6		224.3	4.9	3.8
	AA894345	Similar to MAT1 gene			20.5	2.2	1.2	2.0	2.3	2.1		172.0	2.0	0.9	0.9	1.0	0.8		112.0	2.1	1.8
	M83107_g_at	Transgelin (SM22-alpha)	Tagln	M83107	29.3	3.1	1.6	3.4	5.1	4.6		275.9	8.2	5.3	4.2	7.5	7.2		81.7	14.7	10.0
Group 2	L03294_g_at	Lipoprotein lipase	Lpl	L03294	22.5	1.2	1.6	1.5	2.3	4.5		130.8	0.5	1.0	1.5	2.2	4.4		76.9	3.5	7.8
	rc_AI012030_at	Matrix Gla protein	Mgp	AI012030	120.4	1.0	0.9	1.5	2.6	5.3		1246.7	0.3	0.5	0.6	1.6	2.7		236.2	2.5	4.3
	M80829_at	Troponin T2, cardiac	Tnnt2	M80829	15.7	1.2	0.7	1.1	1.8	2.6		17.3	0.8	2.2	3.3	7.7	17.5		14.9	18.4	15.5
	M22400	Glypican 3/developmentally regulated intestinal protein (OCI-5)	GPC3	M22400	3.7	0.9	2.4	3.9	7.4	10.0		61.9	0.4	0.9	1.5	3.1	9.9		8.6	0.6	1.0
	D00680_at	Glutathione peroxidase 3/plasma glutathione peroxidase precursor	Gpx3	D00680	20.5	1.4	1.5	2.7	3.4	3.9		134.5	0.7	1.8	3.9	9.2	9.2		104.3	5.1	4.3
	rc_AA800844_s_at	Similar to Loxl protein/Loxl1=lysyl oxidase-like 1	Loxl1	AA800844	29.3	1.5	1.5	4.8	7.5	8.5		436.8	0.8	1.4	2.1	3.0	2.5		166.0	3.5	5.3
	S77494_s_at	Lysyl oxidase	Lox	S77494	27.5	0.8	0.8	1.9	2.4	3.0		183.1	1.0	3.2	3.4	4.3	3.1		26.3	29.1	38.0

Group	Probe ID	Annotation	Symbol	Gen Bank	Whole liver						Isolated HSC						In vitro cultured HSC		
					d 0	4	7	14	21	28	d 0	4	7	14	21	28	d 0	3	7
	AF030358 g at	Small inducible cytokine subfamily D, number1/ chemokine CX3C motif, ligand 1	CX3CL1	AF030358	4.6	1.0	2.2	2.3	3.0	4.0							24.0	0.6	1.5
	X84039_at	Lumican	Lum	X84039	8.1	1.0	2.1	2.1	2.3	1.1	91.5	0.3	0.8	2.4	1.5	0.3	52.8	2.3	3.2
	U09540_g_at	Cytochrome P450, Cyp1b1 family 1, subfamily b, polypeptide 1	Cyp1b1	U09540	8.1	1.0	1.3	1.2	1.9	1.6	26.5	4.9	0.8	3.9	10.2	14.6	29.9	5.0	6.2
Group 3	M31038	Non-RT1 class Ib/ MHC class I non-RT1.A alpha-1-chain		M31038	42.2	1.8	4.0	6.5	3.2	3.9	38.3	2.0	10.1	5.0	8.9	0.8	40.7	0.7	0.8
	U44948	Cysteine-and glycine rich-protein 2/smooth muscle cell LIM protein (SmLIM)	CSRP2	U44948	41.9	2.2	1.2	1.6	1.1	1.2	406.7	2.6	0.4	0.4	0.5	0.4	202.0	2.9	1.7
	X02601 at	Matrix metalloproteinase 3		X0260	8.6	1.3	2.0	1.0	0.9	1.3							597.2	1.1	0.1
Group 4	M15880_at	Neuropeptide Y	Npy	M15880	49.2	4.2	1.0	1.2	1.3	0.8	63.1	2.0	0.7	0.2	1.7	0.3	6.2	0.8	6.6
	U50736	Cardiac adriamycin-responsive protein	CARP	U50736	14.3	1.9	1.0	1.0	1.2	1.1	33.7	8.1	2.6	1.6	3.0	4.0	63.6	2.4	1.0

Expression profiles of the whole liver, of isolated HSCs during fibrogenesis, and of in vitro cultured isolated HSCs were obtained using a rat Genome U34A Array (Affymetrix). Marker genes for HSCs are listed. Expression intensities are shown for d 0, and expression intensity data for d 4, 7, 14, 21 and 28 are displayed as ratios to the d 0 expression levels for the analysis of whole liver and isolated HSCs. For in vitro cultured HSCs, the d 0 data are similarly shown as expression intensities, and data on d 4 and 7 are shown as ratios to the expression levels on d 0. Italics indicate an "absent" call by the Affymetrix software, and bold text indicates the highest ratio in the chronic phase (d 7, 14, 21 and 28). □ between 0.667 and 1.5; ■ ≥ 1.5; ■ ≤ 0.667.

drawback, the marker genes could be used to study the behavior of inflammatory cells during fibrogenesis, and these genes were therefore defined as inflammatory cell-specific marker genes. The behavior of the inflammatory cell-specific marker genes *in vivo* during fibrogenesis was analyzed using DNA microarray data for the whole liver at each time point during DMN-induced fibrogenesis of marker genes, as summarized in Figures 4 and 5.

The acute phase response was followed by an immunological response, based on the increase in expression of Kupffer cell (or macrophage) markers such as Lyz, Gzmb, and Il1b, as well as surface markers of T cells, such as T-cell receptor, Il2rb, Cd8, Cd76, and Cd45. The up-regulated expression of these genes seemed to indicate activation of Kupffer cells and T lymphocytes, as well as activation of the interaction between these cells, around d 7. Temporary up-regulation of mast cell markers such as chemokines and mast cell proteases indicated the invasion and/or activation of mast cells around d 14, which is of interest since mast cells are known not only to cause acute inflammation, but also to have a role in the induction of chronic inflammation^[12-14]. However, whether activation of mast cells is essential for liver fibrosis is unknown. Peak expression of B cell markers such as immunoglobulin occurred on d 21 or d 28, and therefore activation or invasion of B cells seemed to reach its peak in the late phase. Overall, these results showed that inflammatory cell-specific marker genes could be used to monitor the transition of active inflammatory cell populations in fibrosis, and this sequential activation or invasion of inflammatory cells might be related to the stage of fibrotic progression.

Marker genes indicating damage to hepatocytes

Hepatocytes were separated from other hepatic cells at each time point during the course of DMN-induced fibrogenesis, and marker genes expressed mainly in hepatocytes were selected by DNA microarray analysis. The behavior of hepatocyte-specific marker genes *in vivo* during fibrogenesis was analyzed using DNA microarray data for the whole liver at each time point during fibrosis development. Marker genes were categorized based on their functions, as shown in Figure 6. Many abnormally expressed genes were identified and temporal analysis revealed groups of genes showing consistent variation in expression. Gene expression profiles in the chronic phase (d 7, 14, 21 and 28) were clustered into 10 patterns using K-means analysis. Clustered genes with a tendency to temporally decrease (clusters 1, 3 and 10) or increase (clusters 7 and 9) were selected, and then genes were further selected based on a strong correlation coefficient (≥ 0.7 or ≤ -0.7) with fibrosis stage (that is, d 7, 14, 21 and 28). The time courses of expression ratios are shown in Figure 7 as supplemental data. The 41 down-regulated and 19 up-regulated genes that were finally selected are shown in Table 2. These genes appeared to have a strong relationship with progression of fibrosis, and might also share common regulatory expression mechanisms.

Down-regulation of the expression of Cdo1 and Csad showed a strong relationship with fibrotic stage, while expression of Gsta2 was simultaneously up-regulated. Down-regulation of other metabolic enzymes in sulfur-containing metabolic pathways is also shown in Figure 6. These data suggested a broad range of abnormalities in sulfur-containing amino acid metabolic pathways in

Table 3 Marker genes for hematopoietic cells in the Kupffer cell fraction

Group	Probe ID	Annotation	Symbol	GenBank	Whole liver						Cell type	Predicted classification	Reference
					D	0	4	7	14	21	28		
1	M34097_at	Granzyme B/natural killer (NK) cell protease 1 (RNKP-1)	Gzmb	M34097	21.4	1.2	2.1	1.1	0.9	0.8	M/L	Kupffer cell, T cell	5
	rc_AA892775_at	Lysozyme	Lyz	AA892775	220.8	9.1	6.4	3.4	5.3	6.4	M	Kupffer cell	6
	Y12009_at	Chemokine, cc motif, receptor 5	Ccr5	Y12009	11.7	1.8	2.6	1.4	1.4	1.2	M/L	T cell	7
	E13732cds_at	Macrophage inflammatory protein-1 alpha receptor/chemokine, CCmotif, receptor 1/RANTES receptor	Ccr1	E13732	9.4	3.8	2.3	1.5	2.2	2.3	M/L	T cell	8
	X13044_g_at	CD74 antigen/invariant polypeptide of major histocompatibility class II antigen-associated	Cd74	X13044	131.7	1.9	13.9	7.8	7.7	5.6	L	B cell	9, 10
	X04139_s_at	Protein kinase C, beta 1	Prkcb1	X04139	14.5	1.5	3.7	2.4	2.2	1.7	M		11
	X03369_s_at	Similar to tubulin, beta	TUBB	X03369	18.6	0.9	2.0	1.8	1.1	1.4	M		12
	M98820_at	Interleukin 1 beta	Il1b	M98820	19.4	1.6	3.6	1.6	1.1	1.1	M	Kupffer cell	13
	U87627_at	Solute carrier family16 (monocarboxylate transporter), member 3	Slc16a3	U87627	24.8	1.9	1.5	0.8	1.2	0.9	M/L		14
	D00403_g_at	Interleukin 1 alpha	Il1a	D00403	27.6	0.6	1.7	0.8	0.5	0.5	M		15
	rc_AI639534_at	Properdin factor, complement/Factor P PROPERDIN P FACTOR, COMPLEMENT; PFC	Pfc	AI639534	68.5	1.2	2.1	1.6	1.1	0.8	M		16
	X03015_at	CD8 antigen, alpha chain	Cd8a	X03015	3.3	8.5	15.7	5.0	9.1	3.8	L	T cell	OMIM
	M18854_at	Similar to T-cell receptor beta-chain/T-cell receptor active beta-chain C-region	---	M18854	22.7	2.0	3.7	2.6	2.6	2.5	L	T cell	
	rc_AA892506_at	Coronin 1A	Coro1a	AA892506	30.5	3.8	4.1	3.7	3.2	3.6	M/L		OMIM
	M55050_at	Interleukin 2 receptor, beta chain	Il2rb	M55050	48.5	0.9	1.5	0.9	0.9	0.8	L	T cell	OMIM
	M30691_at	Ly6-C antigen gene/CD56	Ly6c	M30691	30.2	3.6	5.0	1.6	1.7	1.1	L	T cell, NK cell	17
	rc_AA891302_at	Similar to Ser/Thr kinase (BL44)	---	AA891302	6.9	3.2	5.4	4.5	3.9	4.1	L	B cell	1
	M10072mRNA_s_at	protein tyrosine phosphatase, receptor type,C/CD45	Ptpnc	M10072	8.2	5.9	4.3	3.1	3.3	2.7	M		OMIM
	S74141_s_at	Hemopoietic cell kinase/hck tyrosine kinase	Hck	S74141	42.8	2.3	3.5	2.3	2.5	2.1	M		OMIM
	X52196cds_at	Arachidonate 5-lipoxygenase activating protein	Alox5ap	X52196	34.5	2.3	2.1	1.7	1.7	1.8	M	Kupffer cell	2
	U93306_at	Kinase insert domain protein receptor	Kdr	U93306	31.3	0.5	1.6	1.2	0.8	0.6	M		18
	U55192_at	Inositol polyphosphate-5-phosphatase D	Inpp5d	U55192	11.6	2.1	3.3	2.1	2.1	1.6	L/M/Leu		19
	rc_AI178971_at	Similar to alpha globin/Hemoglobin alpha	(HBA1)	AI178971	141.3	2.4	0.3	0.4	0.5	0.5	E		20
	D86297_at	Aminolevulinic acid synthase 2	Alas2	D86297	131.4	2.7	0.5	0.5	0.8	0.9	E	Entrez gene	
	Y07704_g_at	Best5 protein	Best5	Y07704	33.8	2.9	0.3	0.5	0.5	0.5	"No information"		
	U50412_at	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1	Pik3r1	U50412	43.8	1.3	0.0	0.0	0.5	0.8	M		4
	AB015191_g_at	Rhesus blood group	Rh	AB015191	22.2	1.4	0.3	0.3	0.5	0.6	E	Entrez gene	
	M94918mRNA_f_at	Hemoglobin beta chain complex/beta-globin	Hbb	M94918	3283.0	1.4	0.5	0.5	0.7	0.6	E	OMIM	
	J04793_at	Solute carrier family 4 (anion exchanger), member 1	Slc4a1	J04793	58.0	1.2	0.7	0.6	1.0	0.7	E	Entrez gene	
	U77697_at	Platelet-endothelial cell adhesion molecule/CD31	Pecam	U77697	45.5	0.7	0.6	0.9	0.8	0.8	M/L/E	OMIM	
2	rc_AI009658_at	Chemokine, CC motif, ligand 5/secreted; RANTES	Ccl5	AI009658	33.2	0.1	1.8	3.1	1.4	0.3	L	T cell	OMIM
	rc_AA957923_at	Mast cell protease 2	Mcpt2	AA957923	10.4	1.1	3.3	12.3	8.7	5.8	M	mast cell	26
	U67914_at	Carboxypeptidase A3	Cpa3	U67914	19.3	0.8	1.0	3.5	2.7	1.6	M	mast cell	27

Group	Probe ID	Annotation	Symbol	GenBank	Whole liver						Cell type	Predicted classification	Reference
					D 0	4	7	14	21	28			
	U67911_s_at	Mast cell protease 9 or mast cell protease 8/mast cell protease 8 precursor (RMCP-8)	"Mcpt9/Mcpt8"	U67911	28.9	0.6	2.2	4.7	3.0	2.1	M	mast cell	Entrez gene
	U67908_at	Chymase 1, mast cell	Cma1	U67908	35.2	1.0	1.2	1.8	1.5	1.2	M	mast cell	Entrez gene 29, 31
	rc_AA957003_at	S100 calcium binding protein A8/calgranulin A	S100a8	AA957003	6.5	7.8	3.2	4.2	1.1	1.4	M/Leu		
	L18948_at	S100 calcium binding protein A9/calgranulin B	S100a9	L18948	16.9	5.6	0.9	2.6	1.0	1.1	M/Leu		31
	U31598_s_at	Major histocompatibility complex, class II, DM alpha (RT1.DMa)	Hla-dma	U31598	98.1	1.9	2.5	2.6	2.6	2.2	M/L	Kupffer cell, mast cell	Entrez gene
	U31599_at	Major histocompatibility complex, class II, DM beta (RT1.DMb)	Hla-dmb	U31599	20.7	2.3	4.3	3.5	4.4	2.8	M/L	Kupffer cell, mast cell	Entrez gene
	L06040_s_at	Arachidonate 12-lipoxygenase	Alox12	L06040	99.8	1.7	0.2	0.1	0.3	0.5	Leu/P		OMIM, 35
3	X06916_at	S100 calcium-binding protein A4 Mts1	S100a4	X06916	18.2	7.1	2.7	1.8	2.2	3.1	M/L		36
	M28671_at	Similar to Ig gamma-2B chain C region (rearranged IgG-2b)	---	M28671	14.7	0.6	0.8	1.0	3.3	0.7	L	B cell	
	rc_A1234828_g_at	Immunoglobulin heavy chain, alpha polypeptide	Igha	A1234828	28.8	0.6	1.0	1.7	2.0	2.4	L	B cell	
	X53517_at	CD37 antigen	Cd37	X53517	32.5	2.7	1.9	2.1	2.3	2.3	L	B cell	OMIM
	X58294_at	Carbonic anhydrase 2	Ca2	X58294	63.8	2.0	0.8	1.0	1.6	2.1	M/L/E		37, 38
	AF072411_g_at	cd36 antigen	Cd36	AF072411	67.0	1.3	1.2	0.9	1.2	1.8	M/E		OMIM
	U75689_s_at	Deoxyribonuclease I-like 3/DNase gamma	Dnase1l3	U75689	106.6	0.1	1.1	1.1	0.5	0.2	M		40
	X73371_at	Fc receptor, IgG, low affinity IIb/Fc gamma receptor	Fcgr2b	X73371	190.7	0.5	0.8	1.1	0.7	0.5	M/L/Leu	B cell, mast cell	OMIM
	L04672_s_at	Adrenomedullin receptor	Admr	L04672	147.0	0.5	0.8	0.9	0.7	0.4	L	T cell possibly	17, 4
4	AF041083_at	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	Slc11a1	AF041083	15.1	2.7	0.7	0.9	1.0	0.9	M		Entrez gene
	D14015_g_at	Cyclin E	CCNE1	D14015	27.1	1.5	1.3	0.9	0.9	1.0	M		44
	U31367_at	Myelin and lymphocyte protein /myelin protein MVP17	Mal	U31367	19.9	2.4	1.0	1.1	1.2	0.7	L	T cell	43

Expression profiles of the whole liver and the isolated Kupffer cell fraction during fibrogenesis were obtained using a rat genome U34A array (Affymetrix). Marker genes for hematopoietic cells in the Kupffer cell fraction are listed. Expression intensities are given for d 0, and expression intensity data for d 4, 7, 14, 21 and 28 are shown as ratios to the d 0 expression data for analysis of hematopoietic cells in whole liver. The classification of genes into groups 1-4 and corresponds to the maximum change of expression at the different time points on d 7, 14, 21, 28 and 4, respectively. Italicized values indicate an "absent" call by the Affymetrix software. The bold values indicate the highest or lowest ratio in the chronic phase (d 7, 14, 21 and 28). □ between 0.667 and 1.5; ▤ ≥ 1.5; ▥ ≤ 0.667. Cell types in the inflammatory cell fraction are as follows: M: Monocytes and their progenitors; L: Lymphocytes and their progenitors; E: Erythrocytes and their progenitors; P: Platelets and their progenitors; Leu: Other kinds of leukocytes and their progenitors.

fibrosis. Regarding other genes, Hao2 and Hpcl2 had a role in fatty acid oxidation in peroxisome, while Amacr was associated with beta-oxidation of pristanoyl-CoA and C27-bile acyl-CoAs. The down-regulation of these genes and other metabolic enzymes related to fatty acid oxidation (Figure 6) suggested abnormalities in the fatty acid oxidation process in fibrosis. The expression of Amacr might be related to that of the nuclear receptor subfamily 0, group B, member 2 (Nr0b2), and changes in NrOb2 expression may affect one of the key molecules in cholesterol biosynthesis. Up-regulation of Gk and down-regulation of Pepck1, PC and Slc37a4 suggested abnormalities in gluconeogenesis, glycogen storage and glycolysis, while down-regulation of Ste and Hsd17b2 suggested an abnormality of estrogen metabolism in the liver. Decreased expression of many Cyp drug metabolism enzymes was also found in progression of fibrosis, and

abnormalities in hormonal signaling were suggested by the down-regulation of Inhbe, Ghr and Dio1. These results showed that identification of hepatocyte-specific marker genes could allow analysis of functional changes in fibrosis, and all the identified abnormalities might have major effects on hepatic function.

DISCUSSION

Marker genes for HSCs

Markers of HSCs such as Acta 2, Cryab, Spp1, Prnp, and Pai-1 were strongly up-regulated on d 4, and then quickly decreased in expression following a gradual up-regulation. On the other hand, other HSC markers such as Gpc3, Lox, and Mgp did not show marked up-regulation on d 4, but their expression level increased linearly during fibrogenesis. Therefore, HSCs may be associated with events in two

Table 4 Marker genes in hematopoietic cells in the Kupffer cell fraction

Group	Probe ID	Annotation	Symbol	GenBank	Whole liver						Isolated Kupffer cells						Cell type	Predicted classification	Ref.
					D 0	4	7	14	21	28	d 0	4	7	14	21	28			
1	M34097_at	Granzyme B/natural killer (NK) cell protease 1 (RNKP-1)	Gzmb	M34097	21.4	1.2	2.1	1.1	0.9	0.8	42.1	1.9	7.5	7.6	3.2	1.3	M/L	Kupffer cell, T1 cell	
	rc_AA892775_at	Lysozyme	Lyz	AA892775	220.8	9.1	6.4	3.4	5.3	6.4	1164.3	5.5	2.9	4.1	2.9	3.9	M	Kupffer cell	2,3
	Y12009_at	Chemokine, cc motif, receptor 5	Ccr5	Y12009	11.7	1.8	2.6	1.4	1.4	1.2	29.4	11.9	8.7	6.8	4.5	5.4	M/L	T cell	4
	E13732cds_at	Macrophage inflammatory protein-1 alpha receptor/chemokine, CCmotif, receptor 1/RANTES receptor	Ccr1	E13732	9.4	3.8	2.3	1.5	2.2	2.3	40.6	11.8	1.7	2.3	1.8	4.0	M/L	T cell	5
	X13044_g_at	CD74 antigen/invariant polypeptide of major histocompatibility class II antigen-associated	Cd74	X13044	131.7	1.9	13.9	7.8	7.7	5.6	1672.0	1.9	2.4	3.1	2.3	2.6	L	B cell	2,6,7
	X04139_s_at	Protein kinase C, beta 1	Prkcb1	X04139	14.5	1.5	3.7	2.4	2.2	1.7	33.5	2.2	3.7	4.5	2.5	4.9	M		8
	X03369_s_at	Similar to tubulin, beta	TUBB	X03369	18.6	0.9	2.0	1.8	1.1	1.4	28.6	0.9	2.5	10.5	4.2	5.3	M		9
	M98820_at	Interleukin 1 beta	Il1b	M98820	19.4	1.6	3.6	1.6	1.1	1.1	884.8	6.8	2.7	2.6	2.2	2.8	M	Kupffer cell	10,11,13
	U87627_at	Solute carrier family16 (monocarboxylate transporter), member 3	Slc16a3	U87627	24.8	1.9	1.5	0.8	1.2	0.9	52.0	10.4	2.8	9.3	5.4	3.2	M/L		14,15,16
	D00403_g_at	Interleukin 1 alpha	Il1a	D00403	27.6	0.6	1.7	0.8	0.5	0.5	1395.6	1.9	1.1	0.4	0.4	0.6	M		17,18
	rc_A1639534_at	Properdin factor, complement/Factor P PROPERDIN P FACTOR, COMPLEMENT; PFC	Pfc	A1639534	68.5	1.2	2.1	1.6	1.1	0.8	49.8	11.9	5.1	5.8	8.7	7.3	M		19
	X03015_at	CD8 antigen, alpha chain	Cd8a	X03015	3.3	8.5	15.7	5.0	9.1	3.8	31.3	2.3	4.4	4.5	2.2	0.9	L	T cell	OMIM
	M18854_at	Similar to T-cell receptor beta-chain/T-cell receptor active beta-chain C-region	---	M18854	22.7	2.0	3.7	2.6	2.6	2.5	37.9	1.0	7.1	15.5	9.3	4.3	L	T cell	
	rc_AA892506_at	Coronin 1A	Coro1a	AA892506	30.5	3.8	4.1	3.7	3.2	3.6	216.3	1.4	3.5	5.2	3.8	3.0	M/L		OMIM
	M55050_at	Interleukin 2 receptor, Il2rb beta chain	M55050	M55050	48.5	0.9	1.5	0.9	0.9	0.8	64.7	2.0	4.1	4.1	2.9	1.7	L	T cell	OMIM
	M30691_at	Ly6-C antigen geneLy6c/CD56	geneLy6c	M30691	30.2	3.6	5.0	1.6	1.7	1.1	173.8	3.7	2.1	2.6	1.8	1.4	L	T cell, NK cell	20,22
	rc_AA891302_at	Similar to Ser/Thr kinase (BL44)	---	AA891302	6.9	3.2	5.4	4.5	3.9	4.1	57.3	1.1	2.6	6.9	4.6	5.8	L	B cell	23
	M10072mRNA_s_at	Protein tyrosine phosphatase, receptor type,C/CD45	Ptpcr	M10072	8.2	5.9	4.3	3.1	3.3	2.7	70.6	6.9	4.6	4.7	1.6	4.3	M		24
	S74141_s_at	Hemopoietic cell kinase hck tyrosine kinase	Hck	S74141	42.8	2.3	3.5	2.3	2.5	2.1	114.0	10.6	4.1	4.5	4.2	4.7	M		OMIM
	X52196cds_at	Arachidonate 5-lipoxygenase activating protein	Alox5ap	X52196	34.5	2.3	2.1	1.7	1.7	1.8	82.4	9.1	3.2	8.4	5.3	6.5	M	Kupffer cell	25,26
	U93306_at	kinase insert domainKdr protein receptor	Kdr	U93306	31.3	0.5	1.6	1.2	0.8	0.6	1516.6	0.1	0.2	0.1	0.1	0.1	M		27,28,29
	U55192_at	Inositol polyphosphate-5-phosphatase D	Inpp5d	U55192	11.6	2.1	3.3	2.1	2.1	1.6	56.8	3.3	2.6	4.4	3.4	3.4	L/M/Leu		OMIM
	rc_A1178971_at	Similar to alpha globin/Hemoglobin alpha	(HBA1)	A1178971	141.3	2.4	0.3	0.4	0.5	0.5	18.2	0.8	0.6	0.5	15.6	1.2	E		OMIM
	D86297_at	Aminolevulinic acid synthase 2	Alas2	D86297	131.4	2.7	0.5	0.5	0.8	0.9	29.9	0.2	4.1	5.2	40.9	4.9	E		Entrez gene
	Y07704_g_at	Best5 protein	Best5	Y07704	33.8	2.9	0.3	0.5	0.5	0.5	668.1	0.0	0.2	0.1	0.4	0.2	No information		
	U50412_at	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1	Pik3r1	U50412	43.8	1.3	0.0	0.0	0.5	0.8	5.0	14.0	2.6	0.5	2.1	3.4	M		30,31,32
	AB015191_g_at	Rhesus blood group	Rh	AB015191	22.2	1.4	0.3	0.3	0.5	0.6	14.1	0.5	4.6	4.8	40.7	4.8	E		Entrez gene

Group	Probe ID	Annotation	Symbol	GenBank	Whole liver						Isolated Kupffer cells						Cell type	Predicted classification	Ref.
					D 0	4	7	14	21	28	d 0	4	7	14	21	28			
	M94918mRNA_f_at	Hemoglobin beta chain complex/ beta-globin	Hbb	M94918	3283.0	1.4	0.5	0.5	0.7	0.6	1212.2	0.3	2.0	2.7	4.8	2.9	E		OMIM
	J04793_at	Solute carrier family 4(anion exchanger), member 1	Slc4a1	J04793	58.0	1.2	0.7	0.6	1.0	0.7	29.0	1.4	3.7	3.1	18.6	2.8	E		Entrez gene
	U77697_at	Platelet-endothelial cell adhesion molecule/CD31	Pecam	U77697	45.5	0.7	0.6	0.9	0.8	0.8	821.2	0.3	0.3	0.2	0.3	0.3	M/L/E		OMIM
2	rc_AI009658_at	Chemokine, CC motif, ligand 5/secreted; RANTES	Ccl5	AI009658	33.2	0.1	1.8	3.1	1.4	0.3	456.2	0.5	0.4	1.6	1.1	0.3	L	T cell	OMIM
	rc_AA957923_at	Mast cell protease 2	Mcpt2	AA957923	10.4	1.1	3.3	12.3	8.7	5.8	23.8	2.9	3.8	89.6	45.0	43.7	M	mast cell	33,34
	U67914_at	Carboxypeptidase A3	Cpa3	U67914	19.3	0.8	1.0	3.5	2.7	1.6	24.6	1.4	2.0	31.4	13.7	19.6	M	mast cell	35,36,37
	U67911_s_at	Mast cell protease 9 or "Mcpt9/mast cell protease 8/ mast cell protease 8 precursor (RMCP-8)	"Mcpt9/Mcpt8"	U67911	28.9	0.6	2.2	4.7	3.0	2.1	81.2	1.2	2.9	24.9	13.4	8.4	M	mast cell	Entrez gene
	U67908_at	Chymase 1, mast cell	Cma1	U67908	35.2	1.0	1.2	1.8	1.5	1.2	32.4	1.2	1.4	16.1	11.3	6.7	M	mast cell	Entrez gene
	rc_AA957003_at	S100 calcium binding protein A8/ calgranulin A	S100a8	AA957003	6.5	7.8	3.2	4.2	1.1	1.4	31.6	6.2	2.6	8.9	7.4	1.0	M/Leu		38,39
	L18948_at	S100 calcium binding protein A9/ calgranulin B	S100a9	L18948	16.9	5.6	0.9	2.6	1.0	1.1	48.9	6.7	4.4	8.9	10.7	1.3	M/Leu		40
	U31598_s_at	Major histocompatibility complex, class II, DM alpha (RT1.DMa)	Hla-dma	U31598	98.1	1.9	2.5	2.6	2.6	2.2	172.1	4.5	3.3	4.9	4.3	6.6	M/L	Kupffer cell, mast cell	Entrez gene
	U31599_at	Major histocompatibility complex, class II, DM beta (RT1.DMb)	Hla-dmb	U31599	20.7	2.3	4.3	3.5	4.4	2.8	33.5	8.3	8.7	8.8	14.4	15.5	M/L	Kupffer cell, mast cell	Entrez gene
	L06040_s_at	Arachidonate 12-lipoxygenase	Alox12	L06040	99.8	1.7	0.2	0.1	0.3	0.5	9.2	3.3	3.0	7.6	35.1	2.5	Leu/P		41,42,43
3	X06916_at	S100 calcium-binding protein A4 Mts1	S100a4	X06916	18.2	7.1	2.7	1.8	2.2	3.1	132.0	9.5	1.5	4.0	2.8	2.3	M/L		45
	M28671_at	Similar to Ig gamma-2B chain C region (rearranged IgG-2b)	---	M28671	14.7	0.6	0.8	1.0	3.3	0.7	61.7	3.3	5.8	13.9	3.9	3.8	L	B cell	
	rc_AI234828_g_at	Immunoglobulin heavy chain, alpha polypeptide	Igha	AI234828	28.8	0.6	1.0	1.7	2.0	2.4	905.3	0.5	0.9	0.9	0.4	0.7	L	B cell	
	X53517_at	CD37 antigen	Cd37	X53517	32.5	2.7	1.9	2.1	2.3	2.3	105.9	3.2	1.8	3.6	2.7	2.1	L	B cell	OMIM
	X58294_at	Carbonic anhydrase 2	Ca2	X58294	63.8	2.0	0.8	1.0	1.6	2.1	36.7	0.4	7.2	5.8	35.8	6.3	M/L/E		46,47
	AF072411_g_at	CD36 antigen	Cd36	AF072411	67.0	1.3	1.2	0.9	1.2	1.8	77.9	9.4	4.0	2.8	3.5	4.6	M/E		OMIM
	U75689_s_at	Deoxyribonuclease I-like 3/DNase gamma	Dnase1l3	U75689	106.6	0.1	1.1	1.1	0.5	0.2	2583.7	0.1	0.2	0.2	0.2	0.1	M		48
	X73371_at	Fc receptor, IgG, low affinity IIb/Fc gamma receptor	Fcgr2b	X73371	190.7	0.5	0.8	1.1	0.7	0.5	2913.7	0.5	0.4	0.4	0.4	0.2	M/L/Leu	B cell, mast cell	OMIM
	L04672_s_at	Adrenomedullin receptor	Admr	L04672	147.0	0.5	0.8	0.9	0.7	0.4	2004.8	0.2	0.3	0.2	0.4	0.1	L	T cell possibly	49,50
4	AF041083_at	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	Slc11a1	AF041083	15.1	2.7	0.7	0.9	1.0	0.9	77.2	1.8	1.6	4.6	2.0	2.1	M		Entrez gene
	D14015_g_at	Cyclin E	CCNE1	D14015	27.1	1.5	1.3	0.9	0.9	1.0	37.6	1.4	2.0	1.5	7.6	1.3	M		
	U31367_at	Myelin and lymphocyte protein/ myelin protein MVP17	Mal	U31367	19.9	2.4	1.0	1.1	1.2	0.7	32.2	14.2	2.4	3.9	2.2	1.8	L	T cell	51

Expression profiles of the whole liver and the isolated Kupffer cell fraction during fibrogenesis were obtained using a rat Genome U34A Array (Affymetrix). Marker genes of hematopoietic cells in the Kupffer cell fraction are listed. Expression intensities are shown for d 0, and the expression intensity data for d 4, 7, 14, 21 and 28 are displayed as ratios to the d 0 expression levels. Italics indicate an "absent" call by the Affymetrix software. Bold values indicate the highest or lowest ratio in the chronic phase (d 7, 14, 21 and 28). □ between 0.667 and 1.5; ▤ ≥ 1.5; ▥ ≤ 0.667. The cell types in the inflammatory cell fraction are as follows: M: Monocytes and their progenitors; L: Lymphocytes and their progenitors; E: Erythrocytes and their progenitors; P: Platelets and their progenitors; Leu: Other kinds of leukocytes and their progenitors.

Table 5 References used in the definition of types of hematopoietic cells in the Kupffer cell fraction

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No.	Reference
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The reference numbers correspond to the numbers in Table 4 in the supplementary data.

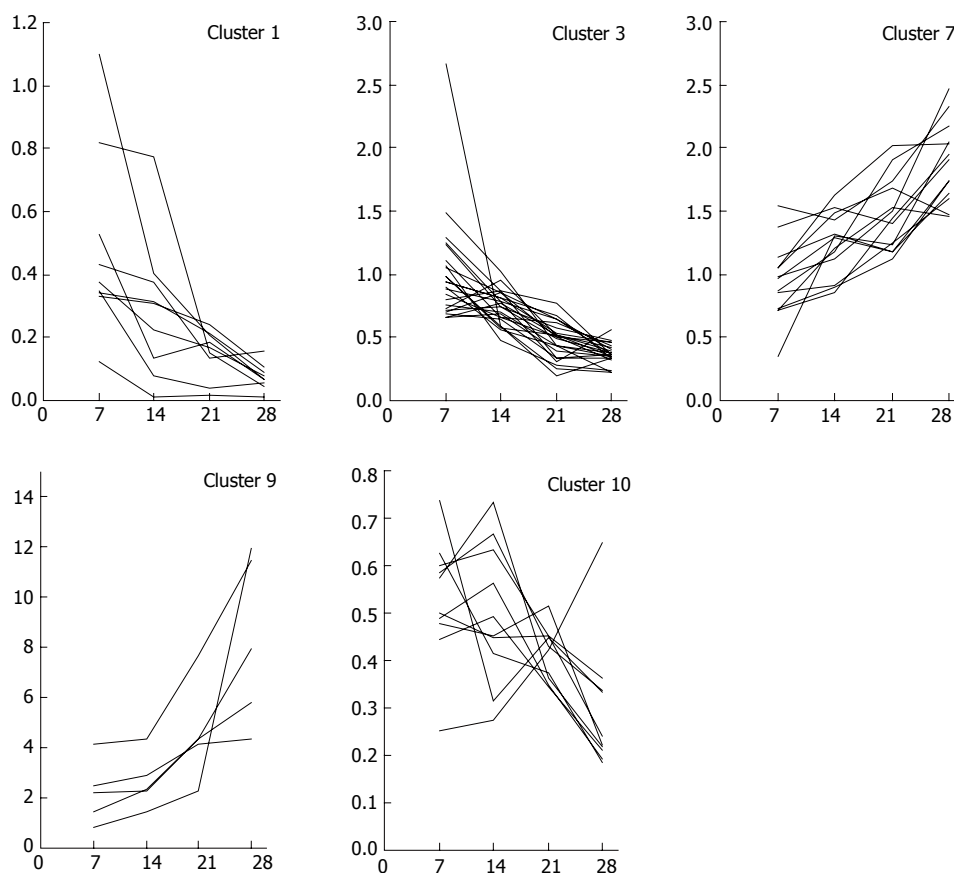


Figure 2 Cluster of genes that increased or decreased in expression with progression of fibrogenesis. Gene expression profiles in the chronic phase (d 7, 14, 21 and 28) were clustered into 10 patterns using K-means analysis. Clustered genes with a tendency to temporally decrease (clusters 1, 3 and 10) or increase (clusters 7 and 9) were selected as gene markers that had a strong relationship with fibrogenesis.

Table 6 Marker genes for hepatocytes

Probe ID	Functional category	Annotation	Symbol	GenBank	UniGene	Whole Liver						Isolated Hepatocytes					
						d 0	4	7	14	21	28	d 0	4	7	14	21	28
U68168_at	Amino acid metabolism	Kynureninase (L-kynurenine hydrolase)	Kynu	U68168	Rn.10575	487.4	0.4	1.0	1.2	0.9	1.2	981.6	0.5	0.8	0.5	0.5	0.4
Z50144_g_at		Aminoadipate aminotransferase	Aadat	Z50144	Rn.11133	336.1	0.4	0.7	0.9	0.9	1.0	360.3	0.6	0.9	0.7	0.6	0.8
AF056031_at		Kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)	Kmo	AF056031	Rn.35029	292.1	0.4	0.9	1.0	0.9	1.1	483.1	0.6	0.5	0.7	0.5	0.5
D44494_at		3-hydroxyanthranilate 3,4-dioxygenase	Haao	D44494	Rn.48675	1071.6	0.5	0.7	0.9	0.7	0.8	948.6	0.6	1.0	0.8	0.8	0.8
M84648mRNA_s_at		Dopa decarboxylase	Ddc	M84648		219.5	0.4	0.5	0.8	0.6	0.6	241.3	0.4	1.1	0.6	0.7	0.3
J02827_at		Branched chain ketoacid dehydrogenase E1, alpha polypeptide	Bckdha	J02827	Rn.3489	194.2	0.5	0.4	0.7	0.7	0.7	164.1	0.7	0.9	0.6	1.0	1.3
rc_AI168942_at		Branched chain keto acid dehydrogenase E1, beta polypeptide	Bckdhb	AI168942	Rn.15623	335.2	0.3	0.6	0.7	0.7	0.7	248.2	0.4	1.0	1.2	1.6	1.9
rc_AI102838_s_at		Isovaleryl coenzyme A dehydrogenase	Ivd	AI102838	Rn.147	345.5	0.4	0.6	0.5	0.7	0.8	439.6	0.6	0.7	0.6	0.7	0.7
M93401_at		Aldehyde dehydrogenase family 6, subfamily A1	Aldh6a1	M93401	Rn.2098	564.1	0.7	0.6	1.2	1.0	1.2	796.3	0.5	0.5	0.8	0.6	0.8
J05499_at		Glutaminase 2 (liver, mitochondrial)	Gls2	J05499	Rn.10202	254.0	0.6	0.9	0.9	0.8	1.3	410.8	0.6	0.4	0.6	0.4	2.1
D10354_s_at		Glutamic pyruvic transaminase 1, soluble	Gpt1	D10354	Rn.6318	229.0	0.6	0.9	0.9	1.2	1.6	201.7	0.9	0.9	1.2	1.0	4.1
J04171_at		Glutamate oxaloacetate transaminase 1	Got1	J04171	Rn.5819	262.9	1.0	0.7	0.9	1.1	1.7	342.5	0.7	0.8	0.8	0.6	4.9
M58308_at		Histidine ammonia lyase	Hal	M58308	Rn.10037	244.9	1.1	1.5	1.4	1.2	2.3	413.3	1.3	0.7	0.6	0.9	2.7
X13119cds_s_at		Serine dehydratase	Sds	X13119		425.9	0.7	0.7	1.1	0.7	1.2	116.7	0.6	0.2	1.2	0.3	23.0
rc_AA892112_g_at		Proline dehydrogenase (oxidase) 2 (predicted)	(Prodh2)	AA892112	Rn.4247	423.3	0.5	0.7	1.0	0.9	0.9	597.2	0.5	0.9	0.7	1.1	1.0
rc_AA892345_at		Dimethylglycine dehydrogenase precursor	Dmgdh	AA892345	Rn.3646	219.5	0.4	0.7	0.8	1.0	0.8	276.6	0.5	0.8	0.7	0.8	0.8
AF067650_at		Sarcosine dehydrogenase	Sardh	AF067650	Rn.37484	133.1	0.7	0.5	0.8	0.6	0.6	112.7	0.5	0.5	0.7	0.6	1.3
J03588_at		Guanidinoacetate methyltransferase	Gamt	J03588	Rn.33890	2017.0	0.3	0.7	0.8	0.6	0.7	1684.6	0.4	1.0	0.8	1.0	0.7
X06150cds_at		Glycine N-methyltransferase	Gnmt	X06150	Rn.11142	605.4	0.2	0.7	0.6	0.5	0.6	801.9	0.3	0.5	0.5	0.3	0.8
X15734_at		Methionine adenosyl-transferase I, alpha	Mat1a	X15734	Rn.10418	1290.3	0.5	0.5	0.6	0.6	0.5	438.8	0.6	0.6	0.6	0.8	2.9
AF038870_at		Betaine-homocysteine methyltransferase	Bhmt	AF038870	Rn.11406	2239.0	0.3	0.7	0.8	0.8	0.8	3346.2	0.3	0.7	0.8	0.7	0.8
M59861_at		Formyltetrahydrofolate dehydrogenase	Fthfd	M59861	Rn.2328	554.2	0.5	0.9	1.3	1.4	1.0	924.3	0.5	1.0	0.9	1.0	1.0
rc_AA942685_at		Cytosolic cysteine dioxygenase 1	Cdo1	AA942685	Rn.2589	1815.9	0.4	0.9	0.8	0.5	0.5	1960.7	0.4	0.6	0.6	0.6	0.5
M64755_at		Cysteine sulfinic acid decarboxylase	Csad	M64755	Rn.43232	394.3	0.1	0.4	0.5	0.3	0.2	403.3	0.2	0.7	0.3	0.6	0.1
rc_AI012802_at		Hydroxyacyl glutathione hydrolase	Hagh	AI012802	Rn.11048	537.6	0.6	0.6	0.9	0.7	0.9	657.2	0.5	0.8	0.7	0.7	0.8

Probe ID	Functional category	Annotation	Symbol	GenBank	UniGene		Whole Liver						Isolated Hepatocytes								
							d 0	4	7	14	21	28	d 0	4	7	14	21	28			
E01415cds_s_at		Glutathione S-transferase, mu type 3	Gstm3	E01415		314.8		0.2	0.7	0.5	0.5	0.6	456.3		0.2	0.4	0.5	0.4	0.5		
rc_AI235747_at		Glutathione transferase YA subunit	Gsta5	AI235747	Rn.10460	77.2		0.6		2.1	1.5	1.2	0.8	94.0		0.7	0.8	1.3	0.8	0.3	
rc_AA945082_at		Glutathione-S-transferase, alpha type2	Gsta2	AA945082	Rn.40574	10.3		2.0	2.2	2.3	4.4	7.9	24.1		1.4	2.5	3.1	2.2	2.6		
K03041mRNA_s_at		Ornithine transcarbamylase	Otc	K03041		219.7		0.4	0.7	1.0	0.8	0.9	259.2		0.4	0.5	0.8	0.4	0.5		
X12459_at		Arginosuccinate synthetase	Ass	X12459	Rn.5078	2507.3		0.6	0.7		0.7	0.8	2828.7		0.3	0.6	0.6	0.5	1.4		
J03959_g_at		Urate oxidase	Uox	J03959	Rn.11330	74.9		0.4	0.4	0.7		0.7	0.6	67.0		0.4	0.9	0.7	1.0	1.0	
M33648_at	Cholesterol synthesis & bile acid synthesis	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	Hmgcs2	M33648	Rn.29594	3672.7		0.4	0.5	0.7		0.5	0.5	3097.5		0.6	0.8		0.9	0.8	
rc_AI180442_at		Farnesyl diphosphate synthase	Fdps	AI180442	Rn.2622	193.8		0.2	0.6	0.6		0.5	0.5	212.0		0.4	1.2	0.8	1.6	0.2	
M95591_at		Farnesyl diphosphate farnesyl transferase 1	Fdft1	M95591	Rn.3252	594.7		0.2	0.3	0.6	0.4	0.5		480.9		0.4	0.8	0.7	0.9	0.2	
AB016800_at		7-dehydrocholesterol reductase	Dhcr7	AB016800	Rn.228	198.3		0.3	0.7	1.1	0.8	1.1		312.6		0.6	0.9	0.8	1.0	0.4	
D86745exon_s_at		Nuclear receptor subfamily 0, group B, member 2	Nr0b2	D86745		201.3		0.2	0.6	0.4	0.4	0.2		147.5		0.4	0.3	0.4	0.6	0.5	
D14989_f_at		Similar to Alcohol sulfotransferase (Hydroxysteroid sulfotransferase) (ST) (ST-60)	LOC361510	D14989	Rn.40365	334.8		0.3	1.0	0.9		0.4	1.4	372.1		1.0	0.7	1.5	0.8	0.5	
D43964_at		Bile acid-Coenzyme A: amino acid N-acyltransferase	Baat	D43964	Rn.11129	743.8		0.5	1.5	1.2	0.9	0.7	1038.5		0.8	0.8		0.6	0.8	0.6	
E12625cds_at		Sterol-C4-methyl oxidase-like	Sc4mol	E12625		579.8		0.1	0.2	0.5	0.2	0.3	452.9		0.1	0.4		0.4	0.8	0.1	
L16995_at		Sterol regulatory element binding factor 1	Srebfl	L16995		116.7		0.7	1.0	1.5	1.2	1.6	227.6		0.2	0.6		1.3	0.7	0.3	
rc_AA866264_s_at	Steroid hormone synthesis & metabolism	Similar to 20-alpha ---hydroxysteroid dehydrogenase		AA866264	Rn.14713	37.8		0.5	1.0	1.1		1.5	2.5	100.8		0.2	0.7	0.8	0.6	1.2	
S76489_s_at		Sulfotransferase, estrogen preferring estrogen sulfotransferase	"Ste, ste2"	S76489		1787.7		0.3	0.7	0.9		0.5	0.3	1919.8		0.3	0.8		0.4	0.3	0.1
J05035_at		Steroid 5 alpha-reductase 1	Srd5a1	J05035	Rn.4620	483.3		0.4	1.1	1.0	0.7	1.1	1175.7		0.6	0.3		0.6	0.8	0.2	
U72497_at		Fatty acid amide hydrolase	Faah	U72497	Rn.10619	493.0		0.5	0.7	0.9	0.7	0.6	439.6		0.5	1.0	0.7	1.1		0.7	
D17310_s_at		3-alpha-hydroxysteroid dehydrogenase	LOC191574	D17310	Rn.10021	440.6		0.5	0.8	0.8		0.6	0.8	576.3		0.8	0.8	0.7	0.7	0.5	
rc_AI105448_at		Hydroxysteroid 11-beta dehydrogenase 1	Hsd11b1	AI105448	Rn.888	2057.5		0.5	0.8	0.7		0.7	0.6	2047.0		0.3	0.9		0.6	0.8	0.7
X91234_at		Hydroxysteroid (17-beta) dehydrogenase 2	Hsd17b2	X91234	Rn.10515	1060.3		0.2	0.8	0.8		0.2	0.0	158.0		0.4	0.8	0.3	1.7	0.5	
rc_AI101743_s_at		Hydroxysteroid (17-beta) dehydrogenase 4	Hsd17b4	AI101743	Rn.2082	151.5		0.9		0.5	1.1	0.7	0.9	267.3		0.5	0.4		0.8	0.5	1.1
U89280_at		Hydroxysteroid (17-beta) dehydrogenase 9	Hsd17b9	U89280	Rn.10857	901.9		0.6	0.7	0.9	1.4	1.9	734.2		1.4	0.7		2.0	1.6	3.3	
rc_AA893495_at		Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antitrypsin), member 6	Serpina6	AA893495	Rn.2374	1880.6		0.2	0.9	0.8		0.6	0.6	2046.0		0.4	0.7		0.6	0.9	0.3

Probe ID	Functional category	Annotation	Symbol	GenBank	UniGene	Whole Liver						Isolated Hepatocytes					
						d 0	4	7	14	21	28	d 0	4	7	14	21	28
S80431_s_at	Lipid biosynthesis, fatty acid metabolism & lipid transport	Aldo-keto reductase family 1, member D1	Akr1d1	S80431		284.2	0.7	0.5	1.4	0.8	1.0	786.7	0.3	0.2	0.8	0.3	0.9
rc_AI172293_at		Sterol-C4-methyl oxidase-like	Sc4mol	AI172293	Rn.7167	677.2	0.2	0.3	0.6	0.3	0.4	694.9	0.2	0.6	0.4	0.9	0.1
U89905_at		Alpha-methylacyl-CoA racemase	Amacr	U89905	Rn.2590	590.0	0.1	0.6	0.7	0.4	0.2	303.5	0.9	0.9	0.5	0.9	0.6
J02749_at		Acetyl-Coenzyme A acyltransferase 1 (peroxisomal 3-oxoacyl-Coenzyme A thiolase)	Acaa1	J02749	Rn.8913	130.3	0.4	0.8	1.4	0.9	1.7	79.4	2.1	2.3	1.9	2.4	3.8
X95189_at		Acyl-Coenzyme A oxidase 2, branched chain	Acox2	X95189	Rn.10622	510.4	0.2	0.7	1.2	0.5	0.3	797.7	0.2	0.3	0.5	0.6	0.4
X95188_at		Acyl-Coenzyme A oxidase 3, pristanoyl	Acox3	X95188	Rn.10546	73.8	0.5	0.4	0.7	0.6	0.4	56.7	0.4	0.4	0.7	0.9	1.0
K03249_at		Enoyl-Coenzyme A hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	Ehhadh	K03249	Rn.3671	198.2	0.2	0.9	1.0	0.8	0.8	372.1	0.4	0.7	0.9	0.9	0.3
U64451_at		Acyl-Coenzyme A dehydrogenase, short/ branched chain	Acadsb	U64451	Rn.44423	91.6	0.6	0.6	0.7	0.5	0.6	82.9	0.5	0.1	0.6	0.2	0.9
AF044574_g_at		2-4-dienoyl-Coenzyme A reductase 2, peroxisomal	Decr2	AF044574	Rn.7879	212.3	0.5	1.0	1.4	0.9	1.5	399.5	0.9	1.0	0.7	0.8	0.6
rc_AA893239_at		2-hydroxy-phytanoyl-Coenzyme A lyase	Hpcl2	AA893239	Rn.21425	480.5	0.1	0.4	0.4	0.1	0.2	236.1	0.5	0.3	0.3	0.4	0.6
rc_AI175764_s_at		Stearoyl-Coenzyme A desaturase 1	Scd1	AI175764	Rn.1023	52.7	0.2	0.7	1.5	1.7	0.8	47.2	0.1	2.6	5.3	2.3	0.1
rc_AA893242_g_at		Acyl-CoA synthetase long-chain family member 1	Acs1l	AA893242	Rn.6215	415.1	0.3	0.5	1.0	0.7	0.8	822.4	0.2	0.3	0.6	0.4	0.4
rc_AI171506_at		Malic enzyme 1	Me1	AI171506	Rn.3519	39.6	0.6	1.1	1.4	0.9	1.8	52.7	0.8	1.7	1.1	1.8	0.3
X70223_at		Peroxisomal membrane protein 2	Pxmp2	X70223	Rn.10292	1059.1	0.3	0.8	0.7	0.6	0.6	907.9	0.7	0.9	0.8	1.0	0.5
rc_AI232087_at		Hydroxyacid oxidase 2 (long chain)	Hao2	AI232087	Rn.10417	731.5	0.4	1.2	0.7	0.6	0.5	693.5	0.2	0.9	0.4	0.3	0.4
U10697_s_at		Carboxylesterase 1	Ces1	U10697	Rn.82692	574.2	0.4	1.2	0.8	0.7	0.6	691.0	0.9	0.9	0.6	1.1	0.4
M20629_s_at		Esterase 2	Es2	M20629	Rn.2549	2513.3	0.2	0.6	0.6	0.5	0.2	2429.2	0.5	0.7	0.6	1.0	0.0
AB010635_s_at		Carboxylesterase 2 (intestine, liver)	Ces2	AB010635	Rn.14535	42.1	9.7	4.2	4.4	7.7	11.5	45.3	15.2	4.9	7.0	10.1	15.6
L46791_at		Carboxylesterase 3	Ces3	L46791	Rn.34885	223.0	0.1	1.0	0.6	0.5	0.4	463.6	0.1	0.8	0.4	0.6	0.0
M16235_at		Lipase, hepatic	Lipc	M16235	Rn.1195	760.7	0.4	0.7	0.9	0.7	0.6	826.2	0.6	0.7	0.9	1.1	0.5
X03468_at		Apolipoprotein A- II	Apoa2	X03468	Rn.10309	2543.4	0.3	0.8	0.6	0.8	0.7	3332.3	0.5	0.6	0.4	0.7	0.2
M00002_at		Apolipoprotein A-IV	Apoa4	M00002	Rn.15739	298.8	0.4	0.6	0.6	0.5	0.6	411.7	0.6	0.7	0.2	0.8	0.5
U53873cds_at	Retinoid synthesis & metabolism	Apolipoprotein B	Apob	U53873		731.2	0.8	0.2	0.4	0.3	0.2	246.2	0.1	0.1	0.7	0.0	1.2
rc_AA945171_at		Apolipoprotein C-IV	Apoc4	AA945171	Rn.33157	720.3	0.7	0.6	0.9	0.7	0.8	1066.0	0.6	0.8	0.5	0.5	0.7
rc_AA893213_at		Apolipoprotein M	Apom	AA893213	Rn.262	1525.1	0.6	0.7	0.8	0.7	0.8	1912.6	0.8	0.9	0.6	0.8	0.6
U02096_at		Fatty acid binding Protein 7, brain	Fabp7	U02096	Rn.10014	190.4	0.3	1.1	0.5	0.3	0.2	129.4	0.7	0.8	0.3	0.3	0.1
U26033_at		Carnitine O-octanoyl-transferase	Crot	U26033	Rn.4896	100.7	0.9	1.7	2.3	1.9	2.0	142.9	1.9	2.2	2.8	1.1	1.9
K03045cds_r_at		Retinol binding protein 4, plasma	Rbp4	K03045		3156.1	0.6	0.6	0.6	0.6	0.4	2863.7	0.5	0.6	0.6	0.9	0.6
U33500_g_at		Retinol dehydrogenase type II (RODH II)	RoDHII	U33500	Rn.37873	255.1	0.7	1.3	0.9	0.8	0.4	95.0	0.8	2.1	0.4	0.7	3.2
U18762_at		Retinol dehydrogenase type III	Rdh3	U18762	Rn.31786	72.9	0.2	0.3	0.3	0.4	0.6	110.2	0.5	0.3	0.8	0.9	0.3

Probe ID	Functional category	Annotation	Symbol	GenBank	UniGene	Whole Liver						Isolated Hepatocytes					
						d 0	4	7	14	21	28	d 0	4	7	14	21	28
X53588_at	Glucolysis & Gluconeogenesis	Glucokinase	Gck	X53588	Rn.10447	56.7	0.5	0.4	1.3	1.2	2.0	218.3	0.1	0.3	0.3	0.1	0.1
M86235_at		Ketohexokinase	Khk	M86235		813.7	0.4	0.8	1.0	0.8	0.9	701.4	0.5	1.4	1.0	1.3	0.9
rc_AA945442_at		Glucokinase regulatory protein	Gckr	AA945442	Rn.7863	248.0	0.3	0.8	1.2	1.0	1.0	449.8	0.4	1.0	0.6	0.8	0.4
rc_AA892395_s_at		Aldolase B	Aldob	AA892395	Rn.10592	2821.0	0.5	0.8	0.9	0.7	0.8	3179.1	0.6	0.8	0.7	0.7	0.9
AB002558_at		Glycerol-3-phosphate dehydrogenase 1 (soluble)	Gpd1	AB002558	Rn.44452	164.6	0.9	0.7	1.1	0.7	0.7	102.5	0.6	0.9	1.5	0.9	1.0
X05684_at		Pyruvate kinase, liver and RBC	Pklr	X05684		111.0	0.2	0.8	0.8	0.8	0.4	107.4	0.3	1.1	0.7	0.9	0.2
U32314_at		Pyruvate carboxylase	Pc	U32314	Rn.11094	398.3	0.3	0.7	0.8	0.5	0.4	456.4	0.5	0.8	0.5	0.8	0.5
X15580complete_seq_s_at		6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1	Pfkfb1	X15580	Rn.10115	262.8	0.1	0.3	0.4	0.5	0.4	89.2	0.4	1.0	0.5	0.8	0.7
rc_AA892799_i_at		Glyoxylate reductase/hydroxypyruvate reductase (predicted)	(Grhpr)	AA892799	Rn.7815	669.4	0.4	0.4	1.0	0.7	0.7	561.6	0.4	0.8	0.7	0.8	0.5
L37333_s_at		Glucose-6-phosphatase, catalytic	G6pc	L37333	Rn.10992	864.9	0.2	0.8	1.2	0.3	0.4	1915.9	0.4	0.5	0.6	1.0	0.6
X04069_at		Liver glycogen phosphorylase	Pygl	X04069	Rn.21399	129.7	0.5	1.0	1.4	1.1	0.8	248.9	0.5	0.8	0.7	0.7	0.3
K03243mRNA_s_at		Phosphoenolpyruvate carboxykinase 1	Pck1	K03243		2955.1	0.5	0.7	0.6	0.3	0.3	2764.6	0.9	0.3	0.5	0.9	1.0
AF080468_at		Solute carrier family 37 (glycerol-6-phosphate transporter), member 4	Slc37a4	AF080468	Rn.1592	681.2	0.2	0.7	0.7	0.5	0.4	609.2	0.4	0.7	0.6	1.0	0.4
rc_AI030175_s_at		Sorbitol dehydrogenase	Sord	AI030175	Rn.11334	762.4	0.7	0.7	1.1	0.9	0.8	1108.8	0.5	0.6	0.8	0.7	0.6
D63704_g_at	Nucleotide-related enzymes	Dihydropyrimidinase	Dpys	D63704	Rn.10586	388.7	0.4	0.7	0.7	0.5	0.5	466.5	0.6	0.7	0.7	0.8	0.3
M97662_at		Ureidopropionase, beta	Upb1	M97662	Rn.11110	1487.6	0.4	0.5	0.7	0.9	0.7	1151.1	0.4	1.0	0.8	1.1	0.8
D85035_at		Dihydropyrimidine dehydrogenase	Dpyd	D85035	Rn.17564	230.4	0.3	0.8	0.7	0.8	1.0	238.1	0.5	0.9	0.9	0.5	1.7
AF041066_at		Ribonuclease, RNase A family 4	Rnase4	AF041066	Rn.22804	1617.5	0.3	1.0	0.9	0.6	0.4	1395.3	0.5	0.8	0.8	1.1	0.6
M57507_at		Guanylate cyclase 1, soluble, beta 2	Gucy1b2	M57507	Rn.10933	85.8	0.3	0.5	0.8	0.5	0.6	95.5	0.3	0.9	0.4	0.5	0.3
E01184cds_s_at	Drug-metabolism	Cytochrome P450, family 1, subfamily a, polypeptide 2	Cyp1a2	E01184		1518.5	0.1	0.3	0.3	0.2	0.1	1898.4	0.4	0.4	0.3	0.4	0.0
J04187_at		Cytochrome P450, subfamily 2A, polypeptide 1	Cyp2a2	J04187	Rn.9867	798.2	0.5	0.8	0.8	0.7	0.6	1022.0	0.4	0.8	0.6	0.6	0.5
K01721mRNA_s_at		Cytochrome P450, family 2, subfamily b, polypeptide 15	Cyp2b15	K01721	Rn.2287	462.6	0.2	0.7	0.4	0.3	0.7	1031.8	0.4	0.3	0.5	0.2	0.1
X79081mRNA_f_at		Cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase)	Cyp2c	X79081		591.2	0.0	0.4	0.2	0.2	0.1	411.3	0.1	0.2	0.1	0.1	0.1
J02861mRNA_s_at		Cytochrome P450 2c13	Cyp2c13	J02861	Rn.32070	1659.9	0.5	1.6	1.0	0.8	0.6	1914.7	0.5	0.9	0.7	0.6	0.7
AB008424_s_at		Cytochrome P450, family 2, subfamily d, polypeptide 13	Cyp2d13	AB008424	Rn.32106	1316.3	0.4	0.9	1.0	0.7	0.7	1998.3	0.5	0.8	0.5	0.8	0.5
AB008423_s_at		Cytochrome P450, family 2, subfamily d, polypeptide 26	Cyp2d26	AB008423	Rn.40137	3830.8	0.7	1.0	0.7	0.7	0.6	4520.5	0.7	0.7	0.6	0.8	0.7
S48325_s_at		Cytochrome P450, family 2, subfamily e, polypeptide 1	Cyp2e1	S48325		5249.5	0.4	0.6	0.5	0.5	0.5	6219.9	0.4	0.5	0.5	0.6	0.6

Probe ID	Functional category	Annotation	Symbol	GenBank	UniGene	Whole Liver						Isolated Hepatocytes					
						d 0	4	7	14	21	28	d 0	4	7	14	21	28
AF017393_at		Cytochrome P450, family 2, subfamily f, polypeptide 2	Cyp2f2	AF017393	Rn.10817	180.5	0.5	0.7	1.0	0.9	1.0	234.2	0.8	0.9	0.7	1.0	0.5
U40004_s_at		Cytochrome P450, family 2, subfamily j, polypeptide 9	Cyp2j9	U40004	Rn.37480	336.9	0.3	0.7	0.5	0.5	0.4	440.5	0.5	0.7	0.6	0.9	0.3
X62086mRNA_s_at		"Cytochrome P450, subfamily 3A, polypeptide 3 cytochrome P450, family 3, subfamily a, polypeptide 11 cytochrome P-450PCN (PNCN inducible)"	Cyp3a3, Cyp3a11, RGD:628626	X62086		3742.3	0.3	0.5	0.5	0.4	0.5	3344.0	0.5	0.5	0.6	0.4	0.6
M13646_s_at		Cytochrome P450, family 3, subfamily a, polypeptide 11	Cyp3a11	M13646	Rn.37424	2198.3	0.4	1.1	0.7	0.5	0.5	2356.8	0.8	0.7	0.6	0.5	0.1
U46118_at		Cytochrome P450, family 3, subfamily a, polypeptide 13	Cyp3a13	U46118	Rn.10489	37.7	0.7	1.5	1.4	1.7	2.3	28.7	2.8	2.2	1.7	2.2	7.3
D38381_s_at		Cytochrome P450, 3a18	RGD:628709	D38381	Rn.32085	444.8	0.6	1.9	1.0	0.9	1.3	682.8	0.8	0.9	0.7	0.4	1.1
U39206_at		Cytochrome P450 4F4	RGD:708363	U39206	Rn.10170	357.7	0.3	0.8	0.7	0.5	0.7	311.9	0.4	0.6	0.8	0.7	0.9
M94548_at		Cytochrome P450, family 4, subfamily F, polypeptide 2	Cyp4f2	M94548	Rn.5722	1637.4	0.6	1.0	0.9	0.8	0.8	2192.8	0.7	0.7	0.6	0.9	0.4
J05460_s_at		Cytochrome P450, family 7, subfamily a, polypeptide 1	Cyp7a1	J05460	Rn.10737	305.0	0.1	1.3	1.5	0.4	0.6	224.3	0.1	0.6	1.3	0.5	0.1
M21208mRNA_s_at		Cytochrome P450, family 17, subfamily a, polypeptide 1	Cyp17a1	M21208	Rn.10172	36.6	0.3	2.7	0.6	0.3	0.2	67.4	0.9	0.5	0.4	0.5	1.7
U17697_s_at		Cytochrome P450, subfamily 51	Cyp51	U17697	Rn.6150	355.0	0.2	0.5	0.7	0.5	0.5	660.8	0.4	0.9	0.6	1.2	0.1
M13506_at		Liver UDP-glucuronosyltransferase, phenobarbital-inducible form	Udpgtr2	M13506	Rn.9969	198.2	1.7	1.4	0.7	0.9	0.8	424.1	2.7	0.5	0.8	1.1	0.6
D38069exon_s_at		UDP glycosyltransferase 1 family, polypeptide A6	Ugt1a6	D38069		63.8	0.6	0.5	1.4	0.9	0.8	83.9	0.6	0.6	1.7	1.0	0.5
rc_AA818122_f_at		Sulfotransferase family, cytosolic, 2A, dehydroepian-drosterone (DHEA)-preferring, member 1	Sth2	AA818122	Rn.2151?	1199.3	0.3	1.3	0.8	0.3	0.6	1674.5	0.9	0.3	0.7	0.5	0.3
L22339_g_at		Sulfotransferase family 1A, member 2	Sult1a2	L22339	Rn.9937	1303.6	0.4	1.1	0.9	0.7	0.8	1587.5	0.4	0.9	0.6	0.4	0.3
rc_AA926193_at		Sulfotransferase family, cytosolic, 1C, member 2	RGD:621064	AA926193	Rn.22471	119.3	0.2	0.7	0.5	0.6	0.5	99.8	0.2	1.0	0.5	0.4	0.2
X56228_g_at		Thiosulfate sulfurtransferase	Tst	X56228	Rn.6360	1476.9	0.4	0.5	0.8	0.7	0.7	1565.7	0.4	0.8	0.6	0.8	0.5
rc_AA892821_at		Aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase)	RGD:620311	AA892821	Rn.8548	184.3	0.4	0.7	1.0	0.8	0.8	223.4	0.6	1.0	0.6	0.6	0.8
AF045464_s_at		Aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)	Akr7a3	AF045464	Rn.6043	337.0	1.5	2.0	1.6	1.4	1.7	240.8	2.9	2.1	1.6	2.0	2.6

Probe ID	Functional category	Annotation	Symbol	GenBank	UniGene	Whole Liver						Isolated Hepatocytes					
						d 0	4	7	14	21	28	d 0	4	7	14	21	28
X65083cds_at		Epoxide hydrolase 2, cytoplasmic	Ephx2	X65083	Rn.54495	44.5	0.8	0.8	1.6	0.6	0.2	22.3	2.6	2.5	1.3	0.2	4.3
AF001898_at		Aldehyde dehydrogenase family 1, member A1	Aldh1a1	AF001898	Rn.6132	304.5	4.1	2.5	2.9	4.1	4.4	752.5	2.2	1.4	1.2	1.5	2.9
M23995_g_at		Aldehyde dehydrogenase family 1, subfamily A4	Aldh1a4	M23995	Rn.74044	167.4	2.1	0.8	0.1	1.2	3.1	148.8	2.1	1.3	5.0	1.6	2.1
rc_AI172017_at		Aldehyde dehydrogenase 2	Aldh2	AI172017	Rn.2300	1125.0	0.5	0.7	0.7	0.6	0.6	1227.2	0.6	1.0	0.6	1.1	0.5
X90710_at		Alcohol dehydrogenase 4 (class II), pi polypeptide	Adh4	X90710	Rn.10302	201.0	0.6	1.5	1.0	0.4	0.4	182.8	0.9	0.5	0.5	0.6	0.5
rc_AA817846_at		3-hydroxy-butyrate dehydrogenase (heart, mitochondrial)	Bdh	AA817846	Rn.36635	574.7	0.3	0.9	0.8	0.7	0.3	723.4	0.3	0.4	0.2	0.7	0.3
rc_AA892382_at		Camello-like 1	Cml1	AA892382	Rn.3643	59.8	0.3	0.7	0.7	0.4	0.3	44.2	0.5	0.7	0.5	0.6	0.4
M26125_at		Epoxide hydrolase 1	Ephx1	M26125	Rn.3603	1661.4	1.7	1.8	1.6	1.5	1.7	2083.7	2.3	1.6	1.8	2.2	1.7
M84719_at		Flavin containing monooxygenase 1	Fmo1	M84719	Rn.867	320.7	0.0	0.5	0.1	0.2	0.1	466.1	0.0	0.5	0.1	0.1	0.0
rc_AA817964_s_at		Paraoxonase 1	Pon1	AA817964	Rn.20732	4497.8	0.4	0.8	0.7	0.7	0.6	4715.3	0.5	0.7	0.7	0.8	0.5
M31363mRNA_f_at		Sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1	Sth2	M31363	Rn.2151	2071.8	0.3	1.0	0.6	0.2	0.3	2228.8	0.7	0.3	0.4	0.4	0.3
M11670_at	Anti-oxidant enzymes	Catalase	Cat	M11670	Rn.3001	697.4	0.4	1.4	0.9	0.7	0.7	723.0	0.6	0.8	0.9	0.8	0.5
U94856_at		Paraoxonase 1	Pon1	U94856	Rn.20732	3127.6	0.4	0.7	0.7	0.6	0.6	3370.6	0.5	0.7	0.7	0.8	0.5
M15481_at	Growth factors & their receptors	Insulin-like Growth factor 1	Igf1	M15481	Rn.6282	534.3	0.4	0.3	0.6	0.4	0.4	367.7	0.6	0.7	0.6	0.7	0.6
M58634_at		Insulin-like Growth factor binding protein 1	Igfbp1	M58634	Rn.34026	521.1	1.8	0.7	0.3	0.4	0.3	914.9	1.8	0.3	0.3	0.9	0.9
J04486_at		Insulin-like growth factor binding protein 2	Igfbp2	J04486	Rn.6813	17.4	2.4	1.5	2.3	4.3	5.8	13.1	6.0	3.3	4.9	5.2	14.6
rc_AA924289_s_at		Insulin-like growth factor binding protein, acid labile subunit	Igfals	AA924289	Rn.7327	217.0	0.3	0.6	0.8	0.5	0.6	234.0	0.3	0.5	0.8	0.8	0.4
AF089825_at		Inhibin beta E	Inhbe	AF089825	Rn.30020	156.2	0.3	0.8	0.9	0.3	0.4	147.0	0.6	1.2	0.8	1.1	0.2
S49003_s_at		Growth hormone receptor	Ghr	S49003		1081.0	0.2	0.5	0.4	0.5	0.4	1080.8	0.2	0.7	0.7	0.9	0.4
AF076619_at		Growth factor receptor bound protein 14	Grb14	AF076619	Rn.30028	210.0	0.7	0.6	0.7	0.4	0.6	221.9	0.8	0.8	0.8	0.5	0.6
M32167_g_at		Vascular endothelial growth factor A	Vegfa	M32167	Rn.1923	42.1	1.0	0.6	0.7	0.4	0.5	36.3	0.8	0.4	0.8	0.7	0.7
M37394_at		Epidermal growth factor receptor	Egfr	M37394	Rn.37227	134.6	0.4	0.6	0.7	0.5	0.4	116.6	0.7	0.8	0.6	0.9	0.5
L48060_s_at		Prolactin receptor	Prlr	L48060	Rn.9757	64.0	0.3	0.6	0.8	1.0	1.5	133.3	0.5	0.5	0.8	1.3	1.1
rc_AA892251_at		Arginine vasopressin receptor 1A	Avpr1a	AA892251	Rn.32282	204.4	0.1	0.4	1.0	1.0	0.6	455.0	0.3	0.7	0.6	1.1	0.7
L32132_at		Lipopoly-saccharide binding protein	Lbp	L32132	Rn.48863	54.1	3.1	0.9	1.2	1.9	2.2	39.4	8.1	1.5	1.9	3.1	5.7
L13025UTR#1_f_at		Polymeric immunoglobulin receptor	Pigr	L13025		421.3	0.5	0.5	0.5	0.5	0.7	1974.1	0.6	0.6	0.8	0.8	0.7
D14869_s_at		Prostaglandin E receptor 3 (subtype EP3)	Ptger3	D14869	Rn.10361	72.3	0.7	0.5	0.5	0.3	0.4	46.6	0.4	0.4	0.5	0.5	0.5
K01934mRNA#2_at		Thyroid hormone responsive protein	Thrsp	K01934		1556.7	0.2	0.8	1.0	0.8	0.7	1201.3	0.1	1.1	1.0	1.4	0.1
X57999cds_at		Deiodinase, iodothyronine, type I	Dio1	X57999	Rn.42914	139.8	0.4	0.6	0.7	0.4	0.3	168.8	0.4	0.5	0.2	0.6	0.2

Probe ID	Functional category	Annotation	Symbol	GenBank	UniGene	Whole Liver						Isolated Hepatocytes					
						d 0	4	7	14	21	28	d 0	4	7	14	21	28
X76456cnds_at	Hepatic secretory proteins	Afamin	Afm	X76456		3150.5	0.3	0.8	0.8	0.7	0.5	3565.8	0.5	0.8	0.9	0.9	0.3
X02361_at		Alpha-fetoprotein	Afp	X02361	Rn.9174	46.5	0.4	0.7	1.2	1.5	1.5	35.4	0.5	1.8	1.2	1.3	0.6
rc_AA817854_s_at		Ceruloplasmin	Cp	AA817854	Rn.32777	418.5	0.9	0.5	1.1	0.8	0.8	362.8	1.1	0.9	1.6	0.5	1.1
X86178mRNA_g_at		Alpha-2-glycoprotein 1, zinc	Azgp1	X86178		871.9	0.5	0.7	0.7	0.7	0.8	1252.1	0.5	0.6	0.9	0.8	0.9
M27434_s_at		Alpha-2u globulin PGCL1		LOC259246	M27434	6719.1	0.2	1.1	0.4	0.2	0.1	4046.4	0.6	0.8	0.1	0.5	0.0
J00738_s_at		Alpha-2u globulin PGCL4		RGD:708508	J00738	527.0	0.0	0.1	0.0	0.0	0.0	53.4	0.1	0.2	0.1	0.1	0.2
X51615_at		Pregnancy-zone protein	Pzp	X51615		55.0	0.7	0.9	0.8	0.6	0.8	63.3	0.6	0.6	2.0	0.9	0.4
rc_AA945608_at	Blood-function	Serum amyloid P-component	Sap	AA945608	Rn.1902	1278.3	0.6	0.8	0.7	0.7	0.7	1286.9	0.9	0.9	0.6	0.9	0.6
rc_AI102562_at		Metallothionein	Mt1a	AI102562	Rn.54397	8150.5	0.6	0.5	0.5	0.5	0.2	2209.3	2.2	0.6	0.6	1.2	1.2
X86561cnds#2_at		Fibrinogen, alpha Polypeptide	Fga	X86561		1512.9	0.8	0.5	0.6	0.6	0.5	1223.6	1.8	1.1	1.0	0.9	0.5
D21215cnds_s_at		Coagulation factor X	F10	D21215		641.3	0.4	0.6	0.9	0.7	0.8	850.3	0.6	0.7	0.9	1.0	0.6
U20194_g_at		Complement component 8, beta polypeptide	C8b	U20194	Rn.10152	1279.6	0.5	0.5	0.6	0.5	0.4	954.2	0.7	0.7	0.5	1.0	0.6
M62832_at		Plasminogen	Plg	M62832		1783.6	0.5	0.5	0.7	0.7	0.6	1618.8	0.5	0.8	0.9	1.2	0.5
M12112mRNA#3_s_at		Angiotensinogen	Agt	M12112		780.9	0.5	0.6	0.9	0.8	0.7	1279.3	0.6	1.0	0.7	1.1	0.9
L00117_at	Protease & protease inhibitors	Elastase 1, pancreatic	Ela1	L00117		132.6	0.0	0.3	0.1	0.0	0.1	74.5	0.2	0.3	0.1	0.0	0.0
rc_AI230712_at		Subtilisin-like endoprotease	Pace4	AI230712	Rn.950	63.8	1.0	0.5	0.9	1.2	1.1	78.2	1.2	0.9	0.5	1.0	1.0
AF097723_s_at		Plasma glutamate carboxypeptidase	Pgcp	AF097723	Rn.17112	519.4	0.3	0.6	0.6	0.4	0.4	464.5	0.4	0.8	0.6	0.7	0.4
X70900_at		Hepsin	Hpn	X70900	Rn.11139	799.9	0.5	0.7	0.8	0.7	0.7	792.6	0.6	0.8	0.7	1.0	0.7
rc_AA946503_at		Lipocalin 2	Lcn2	AA946503	Rn.11303	26.4	4.0	0.8	1.4	2.3	12.0	7.1	126.5	5.2	35.9	4.3	157.1
X69834_at		Serine (or cysteine) proteinase inhibitor, clade A, member 3M	Serpina3m	X69834	Rn.10424	875.6	0.5	0.8	0.8	1.0	1.0	752.4	1.2	0.5	1.6	2.6	0.5
D00752_at		Serine protease inhibitor	Spin2a	D00752	Rn.34396	5647.3	0.3	0.8	0.7	0.6	0.4	6388.0	0.4	0.7	0.5	0.8	0.1
M35299_s_at		Serine protease inhibitor, Kazal type 1	Spink1	M35299	Rn.9767	55.7	0.6	0.8	0.7	0.4	0.5	39.6	0.5	0.3	0.7	0.1	1.1
X16273cnds_at		Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	Serpina1	X16273		6888.3	0.6	1.0	0.7	0.6	0.6	6788.4	1.0	0.8	0.6	0.7	0.9
rc_AA893552_at		Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4	Serpina4	AA893552	Rn.11152	813.0	0.4	0.7	0.6	0.7	0.6	809.8	0.5	1.0	0.6	1.0	0.4
M63991_at	Cell surface proteins & structural proteins	Serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antipeptidase, antitrypsin), member 7	Serpina7	M63991	Rn.9948	25.6	1.8	2.2	1.5	3.8	1.6	10.7	7.1	7.9	4.6	1.8	1.6
M22993cnds_s_at		"Murinoglobulin 1 homolog (mouse) alpha-1-inhibitor III similar to Murinoglobulin 1 homolog murinoglobulin 2"	"Mug1, LOC297568, LOC297572, RGD:1302962"	M22993		979.0	0.5	0.3	0.8	0.5	0.5	1427.7	0.1	0.1	0.6	0.2	0.6
V01216_at		Orosomucoid 1	Orm1	V01216	Rn.10295	1695.5	1.5	1.3	1.0	1.2	1.3	1097.2	3.1	1.9	2.3	2.2	4.6
X05023_at		Mannose-binding protein C (liver)	Mbl2	X05023	Rn.9667	107.8	0.5	0.8	1.0	0.8	0.7	179.0	0.6	0.9	0.8	0.9	0.7
AF080507_at		Mannose-binding protein mRNA	---	AF080507		462.7	0.6	1.2	1.3	1.2	1.1	739.8	0.7	1.0	1.2	1.1	0.6
K02817cnds_s_at		Asialoglycoprotein receptor 1	Asgr1	K02817		544.5	0.8	0.6	0.7	0.6	0.6	661.3	1.0	0.8	0.6	0.9	0.7

Probe ID	Functional category	Annotation	Symbol	GenBank	UniGene	Whole Liver						Isolated Hepatocytes					
						d 0	4	7	14	21	28	d 0	4	7	14	21	28
U82612cds_g_at		Fibronectin 1	Fn1	U82612		626.7	1.0	0.4	0.8	0.6	0.5	607.1	0.4	0.4	0.8	0.8	0.6
M81687_at		Syndecan 2	Sdc2	M81687	Rn.11127	438.7	0.4	0.7	0.9	0.7	0.6	542.6	0.6	0.7	0.8	0.7	0.3
AF090134_at		Lin-7 homolog a (C. elegans)	Lin7a	AF090134	Rn.31766	86.2	0.8	0.6	0.9	0.5	0.7	69.9	1.2	1.4	1.0	0.8	0.5
X04070_at		Gap junction membrane channel protein beta 1	Gjb1	X04070	Rn.10444	1214.5	0.5	0.5	0.6	0.6	0.5	1377.4	0.4	0.7	0.6	1.0	0.6
rc_AA799879_at		Synaptogyrin 1	Syngr1	AA799879	Rn.11188	75.1	0.6	0.6	0.7	0.6	0.5	60.0	1.0	0.7	0.6	0.8	1.1
S76054_s_at		Keratin complex 2, basic, gene 8	Krt2-8	S76054		305.6	2.3	1.0	1.3	1.2	1.7	345.3	2.8	1.6	1.6	1.4	2.0
rc_AI072634_at		Keratin complex 1, Krt1-18 acidic, gene 18	Krt1-18	AI072634	Rn.3603	417.8	1.9	1.1	1.3	1.2	1.6	384.1	3.7	2.2	1.8	1.8	3.3
X59864mRNA_at		H19 fetal liver mRNA	H19	X59864		3.3	17.7	7.5	7.8	48.5	21.2	1.3	1.0	52.8	86.2	1.2	1.1
D31662exon#4_s_at	Signal transduction & transcription factors	Regucalcin	Rgn	D31662		865.7	0.2	0.7	1.0	0.5	0.4	863.6	0.2	1.2	0.7	0.6	0.1
rc_AA893485_at		RAB10, member RAS oncogene family	Rab10	AA893485	Rn.65864	90.1	0.5	0.5	1.3	0.7	0.7	68.0	0.3	1.3	0.6	1.0	0.4
U68544_at		Peptidylprolyl isomerase F (cyclophilin F)	Ppif	U68544	Rn.2923	276.1	0.4	0.7	0.9	0.7	1.1	399.5	0.4	0.5	0.5	0.4	1.1
rc_AA799560_at		N-myc downstream regulated gene 2	Ndrp2	AA799560	Rn.3407	799.7	0.4	1.0	1.2	0.8	0.7	1329.5	0.5	0.6	0.6	0.6	0.4
rc_AA891194_s_at		Arg/ Abl-interacting protein ArgBP2	Argbp2	AA891194	Rn.24612	35.9	1.3	2.0	1.4	1.5	2.0	38.5	4.3	2.1	1.9	2.3	1.5
X57133mRNA_at		Rat mRNA for hepatocyte nuclear factor 4	HNF4	X57133		214.9	0.9	0.4	0.5	0.5	0.3	210.3	0.4	0.4	0.6	0.6	0.8
Y14933mRNA_s_at		One cut domain, family member 1	Onecut1	Y14933	Rn.48812	70.3	0.4	0.2	0.6	0.1	0.2	212.2	0.5	0.4	0.4	0.0	0.3
D86745cds_s_at		Nuclear receptor subfamily 0, group B, member 2	Nr0b2	D86745		486.2	0.2	0.9	0.6	0.4	0.2	304.0	0.5	0.4	0.5	0.7	0.6
X12752_at		CCAAT/enhancer binding protein (C/EBP), alpha	Cebpa	X12752		224.3	0.5	0.3	0.5	0.4	0.3	206.1	0.3	0.4	0.5	0.6	0.4
M81855_at	Transporters	ATP-binding cassette, sub-family B (MDR/TAP), member 1	Abcb1	M81855	Rn.82691	3.2	124.3	24.8	40.6	90.6	108.0	9.1	85.3	20.9	34.8	23.8	63.9
AB010466_s_at		ATP-binding cassette, sub-family C (CFTR/MRP), member 6	Abcc6	AB010466	Rn.29976	452.7	0.4	0.8	0.6	0.5	0.6	415.3	0.5	0.7	0.6	0.9	0.5
U53927_at		Solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	Slc7a2	U53927		29.7	1.6	0.7	0.8	0.7	1.0	27.7	1.1	0.6	0.9	0.5	5.2
M77479_at		Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	Slc10a1	M77479	Rn.9913	1054.3	0.2	0.8	0.7	0.4	0.4	1314.8	0.3	0.5	0.5	0.8	0.3
L23413_at		Solute carrier family 26 (sulfate transporter), member 1	Slc26a1	L23413	Rn.10016	456.1	0.5	0.7	0.8	0.5	0.6	426.3	0.6	1.1	0.6	1.2	0.7
U76379_s_at		Solute carrier family 22 (organic cation transporter), member 1	Slc22a1	U76379	Rn.11186	518.4	0.6	0.6	0.6	0.5	0.5	565.6	0.6	0.8	0.5	0.6	0.4
L27651_g_at		Solute carrier family 22 (organic anion transporter), member 7	Slc22a7	L27651	Rn.10009	423.3	0.6	0.7	0.9	0.5	0.7	477.6	0.9	1.3	0.9	1.3	0.6
U88036_at		Solute carrier organic anion transporter family, member 1a4	Slco1a4	U88036	Rn.5641	560.4	0.4	1.1	1.1	0.9	1.0	588.3	0.6	0.9	0.9	0.5	0.6

Probe ID	Functional category	Annotation	Symbol	GenBank	UniGene	Whole Liver						Isolated Hepatocytes					
						d 0	4	7	14	21	28	d 0	4	7	14	21	28
M95762_at		Solute carrier family 6 (neurotransmitter transporter, GABA), member 13	Slc6a13	M95762	Rn.10527	205.2	0.4	0.7	0.7	0.5	0.5	262.9	0.7	0.6	0.4	0.8	0.5
U28504_g_at		Solute carrier family 17 (sodium phosphate), member 1	Slc17a1	U28504	Rn.11150	36.0	0.6	1.1	1.6	2.0	2.0	78.7	0.9	1.2	1.1	1.3	0.5
M64862_at		Solute carrier organic anion transporter family, member 2a1	Slco2a1	M64862	Rn.9671	241.6	0.6	0.6	0.5	0.6	0.5	166.2	0.3	0.4	0.7	0.9	0.5
AB013112_s_at		Aquaporin 9	Aqp9	AB013112		693.4	0.6	0.7	0.8	0.7	0.7	730.2	0.9	1.2	1.2	1.6	0.5
AB005547_at		Aquaporin 8	Aqp8	AB005547	Rn.6315	158.0	1.1	0.9	0.9	0.9	1.6	103.4	1.7	2.5	2.9	3.5	2.1
AB000507_at		Aquaporin 7	Aqp7	AB000507	Rn.11111	35.9	1.8	1.4	1.5	1.4	1.9	13.7	4.0	4.9	5.9	5.1	4.2
rc_AA799645_g_at		FXVD domain-containing ion transport regulator 1	Fxyd1	AA799645	Rn.3828	169.6	0.4	0.6	0.5	0.5	0.4	200.9	0.5	0.3	0.3	0.5	0.5
AF080568_at	Enzymes related phospholipids	Phosphate cytidylyltransferase 2, ethanolamine	Pcyt2	AF080568	Rn.7291	617.9	0.4	0.4	0.8	0.6	0.7	569.7	0.6	0.8	0.6	0.8	0.6
D28560_at		Ectonucleotide pyrophosphatase/phosphodiesterase 2	Enpp2	D28560	Rn.20403	410.9	0.1	0.9	0.8	0.6	0.4	353.6	0.4	0.7	0.7	0.8	0.5
L14441_at		Phosphatidylethanolamine N-methyltransferase	Pemt	L14441	Rn.9875	758.5	0.4	0.8	0.9	0.7	0.6	809.4	0.6	0.9	0.8	1.1	0.5
rc_AA875050_at		(Ethanolamine kinase)	---	AA875050	Rn.65516	533.0	0.4	1.0	0.9	0.7	0.6	421.1	0.6	0.7	0.6	0.9	1.0
D16339_at	Protein related vitamins	Tocopherol (alpha) transfer protein	Ttpa	D16339		597.7	0.4	1.0	1.0	0.8	0.8	498.8	0.8	0.9	0.9	0.4	0.5
D14564cds_s_at		L-gulonolactone oxidase	Gulo	D14564		1483.9	0.2	0.5	0.6	0.3	0.2	1205.8	0.2	0.8	0.5	0.8	0.2
U19485_g_at	Others	Secreted phosphoprotein 2	Spp2	U19485	Rn.84	2059.5	0.6	0.8	0.8	0.7	0.6	2454.4	0.8	0.8	0.7	0.9	0.6
AF022774_g_at		Rabphilin 3A-like (without C2 domains)	Rph3al	AF022774	Rn.10986	39.9	1.4	1.0	1.5	1.7	1.5	28.5	2.4	1.9	1.2	1.7	2.9
rc_AA945050_f_at		Rat senescence marker protein 2A gene, exons 1 and 2	Smp2a	AA945050	Rn.40124	385.7	0.6	0.9	1.2	0.7	1.4	547.7	1.4	0.5	1.8	0.9	1.0
AF062389_at		Kidney-specific protein (KS)	RGD:708383	AF062389	Rn.14875	44.9	1.1	3.0	2.0	1.4	2.0	50.7	0.5	2.2	4.6	3.8	3.3
AF037072_at		Carbonic anhydrase 3	Ca3	AF037072	Rn.1647	1189.5	0.1	0.3	0.3	0.2	0.1	3196.8	0.0	0.4	0.1	0.1	0.0

Expression profiles of whole liver or isolated hepatocytes during fibrogenesis were obtained using a rat Genome U34A Array (Affymetrix). Marker genes for hepatocytes, which are the main contributors to the expression profile of the whole liver, are listed. Expression intensities are given for d 0, and expression intensity data for d 4, 7, 14, 21 and 28 are displayed as ratios to the d 0 expression data. Italics indicate an "absent" call by the Affymetrix software. Genes with the highest or lowest ratio in the chronic phase are shown in bold text. □ between 0.667 and 1.5; ▤ ≥ 1.5; ▥ ≤ 0.667.

different phases, such as repair in the acute phase response and fibrosis in the chronic phase response. These two groups of HSC marker genes may also be differentially regulated. Marker genes in one group may be expressed both in undifferentiated and in differentiated HSCs, while marker genes in the other group may be expressed mainly in differentiated HSCs. The functional changes associated with differentiation of HSCs during fibrosis are not clear. Schnabel *et al*^[15] have temporally divided the activation process of HSCs into an initiation phase and a perpetuation phase, and HSCs in the initiation phase may play a role in wound healing^[16,17] and are then eliminated

by apoptosis^[18,19], although some HSCs in the initiation phase differentiate into cells in the perpetuation phase. The two kinds of marker genes found in our work may be associated with the two phases.

In the present study, gene expression in HSCs *in vivo* was mostly similar to that found *in vitro*, suggesting that HSCs can be activated and produce ECM with few factors contributed by other cell types. Since HSCs also produce auto-stimulating factors such as TGF-beta, chemokines, PDGF, and IGF-1, our results strongly suggest that HSCs have self-supporting properties and few exogenous factors are required for their activation and differentiation. Even if

Table 7 Marker genes for hepatocytes that showed a strong relationship with fibrogenesis

Functional category	Cluster No.	Direction of change	Annotation	Symbol	Common
Amino acid metabolism	7	Increase	Glutamate oxaloacetate transaminase 1	Got1	J04171
	7	Increase	Glutamic-pyruvate transaminase (alanine aminotransferase)	Gpt	D10354
	9	Increase	Glutathione-S-transferase, alpha type2	Gsta2	AA945082
	3	Decrease	Cytosolic cysteine dioxygenase 1	Cdo1	AA942685
	10	Decrease	Cysteine-sulfinate decarboxylase	Csad	M64755
Cholesterol synthesis	3	Decrease	Nuclear receptor subfamily 0, group B, member 2	Nr0b2	D86745
Steroid hormone synthesis	7	Increase	Hydroxysteroid (17-beta) dehydrogenase 9	Hsd17b9	U89280
	7	Increase	(20-alpha-hydroxysteroid dehydrogenase)	---	AA866264
	1	Decrease	Hydroxysteroid (17-beta) dehydrogenase 2	Hsd17b2	X91234
	3	Decrease	Sulfotransferase, estrogen preferring	Ste	S76489
Lipid biosynthesis, metabolism, fatty acid & lipid transport	9	Increase	Carboxylesterase 2 (intestine, liver) (drug metabolism)	Ces2	AB010635
	1	Decrease	2-hydroxyphytanoyl-CoA lyase(peroxisomal) alpha-oxidation	Hpcl2	AA893239
	3	Decrease	Carboxylesterase 3	Ces3	L46791
	3	Decrease	Hydroxyacid oxidase 2 (long chain)(peroxisomal)(alpha-oxidation)	Hao2	AI232087
	3	Decrease	Fatty acid binding protein 7, brain (cytosolic)	Fabp7	U02096
	10	Decrease	Alpha-methylacyl-CoA racemase Peroxisomal)	Amacr	U89905
Retinoid synthesis & metabolism	3	Decrease	Retinol dehydrogenase type II (RODH II)	RoDHII	U33500
	10	Decrease	Retinol dehydrogenase type III	Rdh3	U18762
Dlucolysis & gluconeogenesis	7	Increase	Glucokinase	Gck	X53588
	3	Decrease	Phosphoenolpyruvate carboxykinase 1(PEPCK1) (cytosolic)	Pck1	K03243
	3	Decrease	Pyruvate carboxylase	Pc	U32314
	3	Decrease	Solute carrier family 37 (glycerol-6-phosphate transporter), member 4	Slc37a4	AF080468
	3	Decrease	Ribonuclease, RNase A family 4	Rnase4	AF041066
	3	Decrease	Ectonucleotide pyrophosphatase/phosphodiesterase 2(lysophospholipaseD)	Enpp2	D28560
Drug-metabolism	7	Increase	Cytochrome P450, family 3, subfamily a, polypeptide 13	Cyp3a13	U46118
	9	Increase	Aldehyde dehydrogenase family 1, member A1	Aldh1a1	AF001898
	1	Decrease	Cytochrome P450, family 1, subfamily a, polypeptide 2	Cyp1a2	E01184
	1	Decrease	Cytochrome P450, subfamily II C (mephenytoin 4-hydroxylase)	Cyp2c	X79081
	1	Decrease	Flavin containing monooxygenase 1	Fmo1	M84719
	3	Decrease	Cytochrome P450, family 17, subfamily a, polypeptide 1	Cyp17a1	M21208
	3	Decrease	Cytochrome P450, family 3, subfamily a, polypeptide 11	Cyp3a11	M13646
	3	Decrease	Alcohol dehydrogenase 4 (class II), pi polypeptide	Adh4	X90710
	3	Decrease	3-hydroxybutyrate dehydrogenase (heart, mitochondrial)	Bdh	AA817846
	3	Decrease	Camello-like 1 N-acetyltransferase 8, NAT8)	Cml1	AA892382
	3	Decrease	(hydroxysteroid sulfotransferase)	---	M31363
	3	Decrease	(hydroxysteroid sulfotransferase subunit)	---	AA818122
Growth factors & their receptors	7	Increase	Lipopolysaccharide binding protein	Lbp	L32132
	9	Decrease	Insulin-like growth factor binding protein 2	Igfbp2	J04486
	3	Decrease	Activin beta E	Inhbe	AF089825
	10	Decrease	Growth hormone receptor	Ghr	S49003
	10	Decrease	Insulin-like growth factor binding protein 1	Igfbp1	M58634
	10	Decrease	Deiodinase, iodothyronine, type I	Dio1	X57999
Hepatic secretory proteins	7	Increase	Alpha-fetoprotein	Afp	X02361
	1	Decrease	Alpha-2u globulin PGCL4 /// alpha-2u globulin PGCL2 /// alpha-2u-globulin (L type)/// alpha-2u globulin PGCL1 /// alpha-2u globulin PGCL3	Obp3 /// LOC298109 /// LOC298116 /// LOC259246 /// LOC259244	M27434
	1	Decrease	Alpha-2u globulin PGCL4 (Ppp2r2a protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), alpha isoform)	Obp3	J00738
	10	Decrease	Metallothionein	Mt1a	AI102562
Protease & protease inhibitor	9	Increase	Lipocalin 2	Lcn2	AA946503
	1	Decrease	Elastase 1, pancreatic	Ela1	L00117
	10	Decrease	Esterase 2 (liver carboxylesterase)	Es2	M20629
	3	Decrease	Serine protease inhibitor	Spin2a	D00752
Cell surface proteins & structural proteins	7	Increase	Keratin complex 2, basic, gene 8 (cytokeratin-8)	Krt2-8	S76054
	7	Increase	Similar to cytokeratin(keratin complex 1, acidic, gene 18)	(Krt1-18)	AI072634
Signal transduction	3	Decrease	Regucalcin	Rgn	D31662
Transporters	7	Increase	solute carrier family 17 (sodium phosphate), member 1	Slc17a1	U28504
	7	Increase	Aquaporin 7	Aqp7	AB000507
	9	Increase	ATP-binding cassette, sub-family B (MDR/TAP), member 1	Abcb1	M81855
	3	Decrease	Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	Slc10a1	M77479

Functional category	Cluster No.	Direction of change	Annotation	Symbol	Common
Protein related vitamins	10	Decrease	L-gulono-gamma-lactone oxidase	Gulo	D14564
Others	7	Increase	Rabphilin 3A-like (without C2 domains)	Rph3al	AF022774
	1	Decrease	Carbonic anhydrase 3	Ca3	AF037072

Gene expression profiles in the chronic phase (d 7, 14, 21 and 28) were clustered into 10 patterns using K-means analysis. Clustered genes that showed a tendency to temporally decrease (clusters 1, 3 and 10) or increase (clusters 7 and 9) were selected, as shown in Table 5 in the supplemental data. Among these genes, those showing a strong relation with fibrogenesis were further selected based on a *t*-test statistical analysis of the rate of change in expression intensity and the number of days of fibrosis. Hepatocyte-specific gene markers showing a strong relation with fibrogenesis are listed.

Inflammation induced by virus activation stimulates long-term or weak hepatitis, HSCs may be able to autonomously activate and promote fibrosis, and this property of HSCs may be central to promotion of fibrogenesis. Sancho-Bru *et al.*^[20] have recently reported that a culture model of HSCs could not exactly match the activated phenotype found in DNA microarray analysis of isolated HSCs from cirrhotic human livers, because in culture marker genes for HSCs in the perpetuation phase are predominantly expressed, relative to those in the initiation phase. However, isolation of HSCs may also alter the expression of some genes, especially the expression of genes associated with inflammation, as shown in Figures 2 and 4. Therefore, compared to studies of isolated HSCs, our approach reveals the actual behavior of HSCs *in vivo* during fibrosis.

Gpc3 has been recently proposed as a serum and histochemical marker for hepatocellular carcinoma^[21,22], since it is only weakly expressed in hepatocytes in normal and cirrhotic livers. However, in our study, Gpc3 was expressed in isolated HSCs during fibrogenesis, but weakly in cultured HSCs, suggesting that HSCs require extracellular factors for expression of Gpc3 during fibrogenesis, or that Gpc3-expressing cells with abnormal characteristics may contaminate the HSC fraction. The cell type showing expression of Gpc3 requires further study.

Marker genes for inflammatory cells

In the present study, gene expression in the inflammatory cell-fraction indicated the presence of several kinds of hematopoietic cells in this fraction, leading to some uncertainty in the data from the rat fibrosis model. However, the behavior of mast cells is of note, since temporary up-regulation of mast cell markers such as chemokines and mast cell proteases indicated invasion and/or activation of mast cells around d 14. Invasion of a marked number of mast cells could not be detected with HE staining, and therefore the number of invading mast cells must be small. Mast cells not only cause acute inflammation, but also have a role in induction of chronic inflammation^[12,14], and involvement of mast cells in hepatic fibrosis has been reported^[12,23-25]. RANTES, a chemokine that is produced by T cells and stimulates mast cells^[26,27], showed its peak expression on d 14, as shown in Figure 4. RANTES has been suggested to be a mediator of progression from acute to chronic inflammation in colitis^[28], and further studies are of importance to determine whether RANTES activation of mast cells is essential for liver fibrosis.

Marker genes for hepatocytes

DNA microarray analysis of whole liver in experimental animal models of hepatic fibrosis has been reported^[1,2], and characteristic behavior of hepatocyte-specific marker genes over the time course of fibrogenesis was found in this study. DNA microarray data for the whole liver are similar to those for hepatocytes, since 70% of hepatic cells are hepatocytes^[8,9], and data on d 28 are generally similar to those in previous reports. However, our temporal data indicate progressive abnormal gene expression in fibrogenesis, including in the early phase of fibrogenesis. Furthermore, since our data did not contain genes expressed in other hepatic cells, the hepatocyte-specific gene set allowed examination of the molecular network in hepatocytes.

Clustered abnormalities were found in genes associated with metabolism of sulfur-containing amino acids in this study. Similar abnormalities in metabolism of sulfur-containing amino acids have been reported^[29,30], and an increase in methionine concentration in blood has been found in cirrhosis^[31,32], which may be related to changes in gene expression in sulfur-containing amino acid metabolism. Furthermore, S-adenosylmethionine has an important role in methylation, including the methylation of DNA. Since abnormalities likely induce tumorigenesis due to DNA instability, it is of note that long-term suppression of S-adenosylmethionine synthetase increases the risk of tumorigenesis^[33]. Glycine methyltransferase also has been implicated in DNA instability^[34,35], and long-term suppression of this enzyme also has an associated risk of tumorigenesis during liver fibrosis. A metabolite of methionine, homocysteine, is also suspected as a risk factor for cardiovascular disease^[36,37], and an increase in the concentration of homocysteine has been found in cirrhosis^[38]. Abnormal homocysteine metabolism may also have an important role in the pathogenesis of liver failure, including fatty liver, activation of HSCs (i.e., enhancement of fibrosis), cardiovascular disease, and HCC. Finally, biosynthesis of taurine from cysteine may be suppressed, possibly leading to painful muscle cramps, which are a complication of cirrhosis caused by taurine deficiency^[39]. Therefore, in summary, it is apparent that abnormalities in sulfur-containing amino acid metabolism can result in development of serious diseases.

Down-regulation of enzymes related to beta-oxidation in TAA-induced experimental fibrosis has been reported^[29]. Studies of the Aox knockout mouse^[40] suggest that metabolites of fatty acids in beta oxidation accumulate in

the liver and stimulate PPAR- α , resulting in peroxisome proliferation, and tumorigenesis is a potential risk in long-term administration of PPAR- α agonists and in the Aox-1 knockout mouse^[40,41]. Radical accumulation by blocking metabolic enzymes associated with beta-oxidation has been suggested as one explanation of tumorigenesis in Aox knockout mice, and furthermore, microarray analysis of Aox-deficient mice can show up-regulation of Lcn2, a marker of carcinogenesis^[42]. Interestingly markers such as Lcn2 and Cd36 were up-regulated in hepatocytes during fibrogenesis and in the inflammatory cell fraction in the late phase in our model. Although tumorigenesis due to PPAR α agonist is thought not to occur in humans^[41,43], abnormalities in lipid metabolism during liver fibrosis may have a role in steatofibrosis and/or enhancement of fibrosis and HCC.

Up-regulation of Gck and down-regulation of Pck1 in hepatocytes suggest a decrease in gluconeogenesis, and suppression of gluconeogenesis has been reported in cirrhosis^[44]. A deficiency of Scl37a4 enzyme activity in humans causes glycogen storage disease type 1 (GSD-1) genetic disorders^[45] and suppression of this molecule is associated with hepatic steatosis^[46]. Abnormalities in metabolism and synthesis of sex hormone have also been found in fibrotic liver, and an increased ratio of estrogen to testosterone in serum induces feminization, which is a complication in cirrhotic males^[47]. Abnormality of sex-hormone metabolism is also related to liver malignancies^[48,49].

Down-regulation of Ghr is also found in hepatocytes, and long-term suppression of growth signals may greatly influence fundamental hepatic vitality and produce abnormal hepatic regeneration. Insensitivity to growth hormones in cirrhosis has been reported^[50,51], and administration of growth hormone protects against experimental liver fibrosis^[52]. Rgn, a regulator of calcium signaling, may have an important role in regulation of proliferation and apoptosis of hepatocytes, as well as in formation of HCC^[53,54], and down-regulation of regucalcin in our model suggested a risk of HCC development. An association of Afp, Ste, Mt1a, Lcn2, Abcb1 Cml1 (Nat8) and Ca3 with hepatocellular carcinoma (HCC) formation has also been reported. Accumulation of estrogen in the liver is suspected to promote HCC^[55,56], and NAT8 polymorphism may be related to HCC^[57,58]. Down-regulation of metallothionein^[59-61] and carbonic anhydrase^[62,63], up-regulation of Mdr/Tap^[64] and Lcn^[42,65] occur in HCC, and proliferation of hepatoma cells is suppressed by over-expression of Rgn^[53,66]. The abnormal expression of all these genes in our model is similar to that in HCC, suggesting the importance of understanding whether such changes in gene expression reflect a tumorigenic environment or even promote tumorigenesis.

Differential regulations of genes involved in key events of liver fibrosis

In addition to the expression profiles of cell type specific marker genes discussed above, we here describe how genes involved in key events of liver fibrogenesis are differentially regulated. Gene expression profiles of the whole liver for different functional categories in liver fibrogenesis, such as ECM synthesis/degradation, inflammation and oxidative

stress, are shown in Figure 8. Since both synthesis and degradation of ECM occur simultaneously, both genes are put together. Figure 8 shows that most genes in each category have a common and mutually correlated expression pattern, showing different regulations for different categories. Most genes of ECM synthesis/degradation as shown in Figure 8A have a peak of up-regulation on d 4 and a following gradual up-regulation along with the progression of fibrosis, and interestingly these genes are classified into group 1 of the HSC-specific genes as shown in Figure 2. Another type of genes of ECM synthesis/degradation as shown in Figure 8B have no peak on d 4 and only a gradual up-regulation along with the progression of fibrosis, and are classified into group 2 of the HSC-specific genes as shown in Figure 2. Many genes are also involved in the inflammatory category, and here only some of them are plotted as representative in Figure 8C. Most genes of inflammation have a peak on d 4 or 7 commonly, and are classified in group 1 of the Kupffer cell fraction specific genes as shown in Figure 4. Most genes in the category of oxidative stress in Figure 8D have a minimum expression on d 4, in contrast to the genes of inflammation having a peak on d 4 or 7, and are involved in hepatocyte specific genes as shown in Figure 6.

Gene expression profiles viewed from both cell types and functional categories have made more clear image on how the temporal expression pattern are closely associated in terms of both the cell specificity and functions in liver fibrosis, and are regulated differently in different categories but in a mutually correlated manner within the same category.

Pathological overview of the behavior of HSCs, inflammatory cells, and hepatocytes in fibrogenesis

Our results from gene-expression profiling using hepatic cell-specific marker genes support the hypothesis shown in Figure 9. Comparisons of gene expression in HSCs *in vivo* and *in vitro* strongly suggest that HSCs have self-supporting properties and that few exogenous molecules are required to activate and differentiate HSCs. Hepatocytes have been shown to suffer from serious stress during fibrogenesis, and signals from suppressed hepatocytes, such as radicals, proinflammatory substances and toxic metabolites due to abnormal metabolism, are able to stimulate Kupffer cells and HSCs, leading to subsequent production of HSC-stimulating-factors such as TNF- α and IL-1 by Kupffer cells. Therefore, HSCs, Kupffer cells and other inflammatory cells produce factors such as TGF- β that suppress hepatocyte vitality and result in hepatocyte injury. Sequential activation of inflammatory cells such as lymphocytes and mast cells may be essential in this process, and even if this stimulatory circuit is small in scale during early remission of fibrogenesis, it can be maintained with appropriate stimulation even at long intervals, with small-scale inflammation induced by C-type hepatitis virus propagation. The self-supporting characteristics of HSCs may have a central role in maintenance of this circuit.

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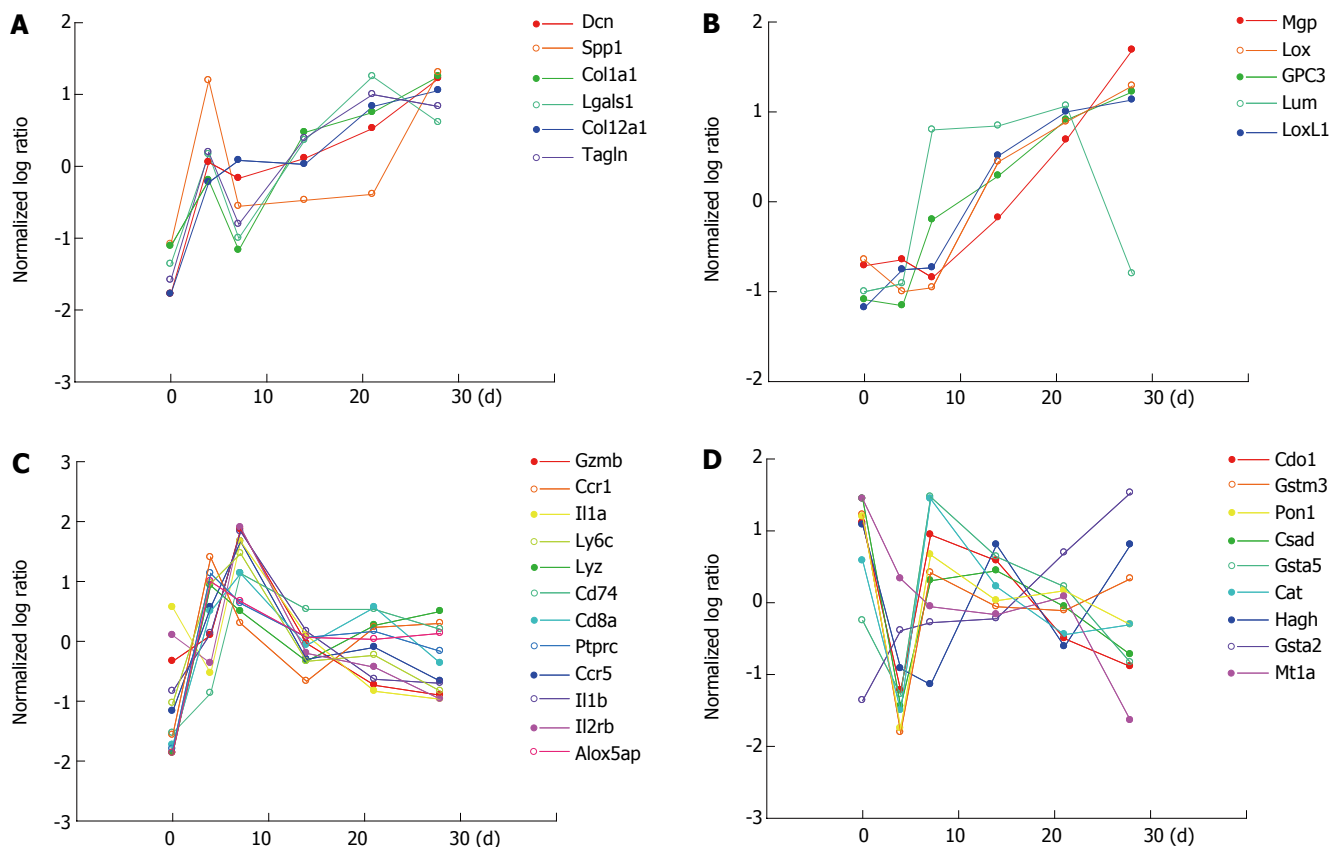


Figure 3 Differential regulations of genes involved in key events of liver fibrosis. Gene expression profiles of different events in liver fibrogenesis such as ECM synthesis/degradation, inflammation and oxidative stress, are shown. The x-axis showing the days of fibrogenesis (d 0, 4, 7, 14, 21, 28) and the y-axis the normalized log ratio (scaled in terms of mean and SD, and the log base 2) of the whole liver gene expression. **A** and **B**: genes of ECM synthesis/degradation classified into group 1 (2) of HSCs as shown in Table 1; **C**: genes of inflammation involved in the Kupffer cell fraction as shown in Table 3; **D**: genes of oxidative stress involved in the hepatocytes as shown in Table 6.

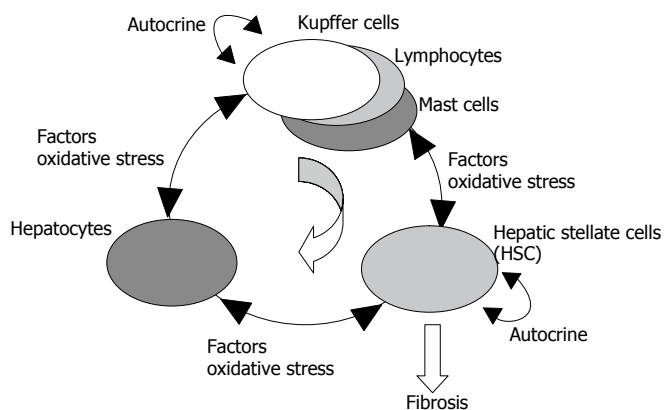


Figure 4 Circuit model of hepatic cells in fibrogenesis. Gene expression profiles show that HSCs have self-activating properties, and that widespread damage to hepatocytes occurred in development of fibrosis, suggesting a self-activating circuit model of fibrogenesis. After an initial stimulatory trigger caused by events such as virus infection, hepatic cells are able to stimulate each other, and the self-activating properties of HSCs maintain this cycle over the long term. Details are given in the text.

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BASIC RESEARCH

Establishment and characterization of a cholangiocarcinoma cell line (RMCCA-1) from a Thai patient

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CONCLUSION: A new cell line derived from peripheral cholangiocarcinoma of a Thai patient has been established. This cell line shows a low level of *in vitro* invasiveness, but a high degree of motility. It will serve as a valuable tool for further studies on tumor biology, molecular pathogenesis, metastatic mechanism and response to therapeutic drugs of cholangiocarcinoma.

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Key words: Cholangiocarcinoma; Cell line; Establishment; mFISH; Invasion; Migration

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Abstract

AIM: To establish and characterize a new cell line derived from peripheral cholangiocarcinoma of a Thai patient.

METHODS: The peripheral cholangiocarcinoma specimen surgically obtained from the patient was aseptically processed by washing and mincing before culturing in Ham's F12 medium containing 10% fetal bovine serum. After 3 mo, when the cell line has become homogeneous and stabilized, several features were investigated, including growth characteristics, immunofluorescence staining for cytokeratins, expression of tumor markers, chromosomal analysis by G-banding and multicolour fluorescence *in situ* hybridization (mFISH), *in vitro* migration and invasion characteristics.

RESULTS: The RMCCA-1 cell line has been established. These cells proliferated as a monolayer with a population doubling time of 48 h. Immunofluorescence staining showed positive staining for human cytokeratin 7 and 19 verifying the biliary epithelial origin. RMCCA-1 secreted carbohydrate antigen 19-9 (CA19-9), but insignificant levels of carcinoembryonic antigen (CEA) and α -fetoprotein (AFP). Chromosome analysis identified aneuploidy karyotypes with a modal chromosome number of 59. RMCCA-1 exhibited a low level of *in vitro* invasiveness, but a high degree of motility. The cell line exhibited a significant number of chromosomal aberrations as shown by mFISH and G-banding methods.

INTRODUCTION

Cholangiocarcinoma is a highly malignant epithelial neoplasm that arises within the intrahepatic and extrahepatic biliary tract^[1]. The pathogenesis of this disease has been strongly associated with chronic inflammation and cellular injury within bile ducts, as well as partial obstruction of bile flow, manifested by various high risk conditions such as PSC (primary sclerosing cholangitis), hepatolithiasis and infestation by liver fluke (*Ophisthorchis viverrini* or *Clonorchis sinensis*)^[2,3]. Although considered as a rare disease, cholangiocarcinoma occurs at a particularly high rate in Northeastern Thailand, with 84.6:100 000 males and 36.8:100 000 females affected by the disease. This is the area where the incidence rate of cholangiocarcinoma is the highest in the world, largely accounted by the habit of consuming uncooked cyprinoid fish, which are infected with the liver fluke^[4].

Cholangiocarcinoma has become a serious threat to public health due to increasing worldwide incidence and mortality rates associated with lack of early detection and limited therapeutic options. At diagnosis, most patients are presented with advanced disease, possibly with undetected metastasis, resulting in less than 12 mo survival. Even those with operable tumor, the recurrence rate was extremely high, with a 5-year survival rate of less

than 40%^[2,5]. Various routes of tumor spreading have been reported in cholangiocarcinoma, including direct invasion, infiltration along the biliary tree, vascular and lymphatic permeation and perineural or intraneural invasion^[6].

Progress in understanding the molecular mechanisms governing cholangiocarcinoma invasion and metastasis has been limited by the lack of suitable cell lines and experimental models. Here, we described an establishment and preliminary characterization of a human cell line originated from a Thai patient presented with peripheral cholangiocarcinoma. This new cell line, which we named RMCCA-1, exhibits various characteristics typical of the biliary epithelial cells, as well as invasiveness and motility as shown by the *in vitro* assay. Thus this cell line will be useful for the studies of not only the tumor biology, molecular pathogenesis and drug response, but also the molecular mechanisms governing the metastatic spread of cholangiocarcinoma.

MATERIALS AND METHODS

Clinical specimen

A 40-year-old male patient was admitted to Rajavithi Hospital, Bangkok, Thailand with a professional diagnosis of peripheral cholangiocarcinoma. The patient's serum was analyzed for alkaline phosphatase (ALP), total bilirubin and direct bilirubin using a COBAS Integra 800 instrument (Roche, USA), whereas CA19-9, carcinoembryonic antigen (CEA), and α -fetoprotein (AFP) were analyzed on a ELECHYS 2012 instrument (Roche, USA). Significant laboratory analysis at admission showed elevated serum levels of ALP (374 U/L, normal 39-117 U/L), total bilirubin (19.73 mg/dL, normal 0.0-1.5 mg/dL), direct bilirubin (15.45 mg/dL, normal 0-0.5 mg/dL), CA19-9 (85.05 U/mL, normal 0-39 U/mL) and CEA (7.56 ng/mL, normal 0.0-3.4 ng/mL), whereas the AFP (2.24 U/mL, normal 0.0-5.8 U/mL), and CA125 (19.71 U/mL, normal 0.0-35 U/mL) levels were normal.

The CT examination evaluated for this study was performed on a helical CT scanner (Somatom Plus 4, Siemens Medical Solutions) using the following parameters: 5-mm collimation, 5-mm reconstruction interval, and a 1:1 table pitch. Both unenhanced and contrast-enhanced CT scans were obtained. With intravenous injection of 120 mL of nonionic contrast material [iopromide (Ultravist 370, Schering)], both hepatic artery phase (HAP) and portal venous phase (PVP) images were obtained with a scanning delay of 30 and 65 s, respectively. The results showed an ill defined hypo-density mass occupying the whole left lobe of the liver. At laparotomy, no ascites was found, and the surface of liver was smooth. Intraoperative ultrasonography revealed a solitary mass at left lobe of liver with moderate dilatation of left intrahepatic duct. The patient was then subjected to left hepatectomy and lymph node dissection. The tumor specimen was removed and subjected to histopathological study and to tissue culture under the approval of the Ethics Committee of Rajavithi Hospital.

Tumor histopathology

The lesion was classified as a well-differentiated peripheral

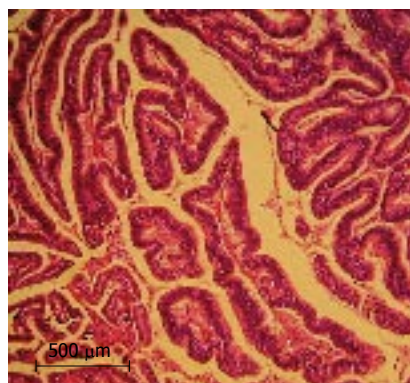


Figure 1 Hematoxylin and Eosin staining of the liver specimen (10 × magnification). The histopathological analysis of the specimen indicates a well-differentiated peripheral cholangiocarcinoma with no vascular invasion.

cholangiocarcinoma at stage T2N0M0 according to the UICC standardization (Figure 1).

Primary culture

After the tumor tissue was surgically removed from the patient, it was immediately suspended in transfer medium [HAM's F12 (GIBCO, Grand Island, NY, USA) containing antibiotics (GIBCO, Grand Island, NY, USA)] at 4°C. The tissue was quickly washed in PBS pH 7.4 several times before being minced. Later the cell suspension was placed in a 100 mm × 20 mm plastic tissue culture dish (CORNING, New York, USA) containing 10 mL of growth medium (HAM's F12, 20% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA), 1×10^{-5} g/L epidermal growth factor (EGF) (Pacific Science, Peprotech, New Jersey, USA), 0.1 U/L penicillin G sodium, 0.1 g/L streptomycin sulfate and 2.5×10^4 g/L amphotericin B). The cell cultures were then incubated at 37°C in a humidified 50 mL/L CO₂ atmosphere and observed daily. Tumor cells were separated from the contaminating fibroblast cells by manually dropping 2.5 mL/L trypsin containing 0.2 mL/L EDTA (GIBCO, Grand Island, NY, USA) in PBS onto an isolated tumor colony. Subsequently the detached tumor cells were transferred by pipettes to a new culture dish under a phase contrast microscope. After about 1 mo, a homogeneous layer of epithelial tumor cells with sustained growth pattern was established.

Growth kinetics

A suspension of 2×10^3 cells was cultured in triplicates in 100 μ L of Ham's F12 medium supplemented with 100 mL/L FBS in a 96-well plate (CORNING, New York, USA). At time intervals, 10 μ L of 5 mg/mL MTT (USB, Cleveland, OH, USA) solution was added to the individual wells, followed by incubation for 4 h at 37°C in a humidified atmosphere containing 50 mL/L CO₂. MTT converted to insoluble formazan dye in live cells was then dissolved by addition of 200 μ L DMSO (Sigma, St. Louis, MO, USA) before the absorbency was read at 540 nm. The doubling time of the cell population was determined from the exponential phase of the growth curve.

Immunofluorescence staining

The monoclonal antibody mixture AE-1/AE-3 (DAKO, Denmark) recognized all known basic and most acidic keratin, thus it was used as a general marker of epithelial cells. Cytokeratin 7 (monoclonal mouse Anti-Human

Cytokeratin 7, DAKO, Denmark) and cytokeratin 19 (monoclonal mouse Anti-Human Cytokeratin 19, DAKO, Denmark) were used to specifically distinguish biliary epithelial cells from hepatocytes^[7,8]. RMCCA-1 cells were grown on sterile coverslips until confluent before being fixed in methanol for 15 min, blocked in 10 mg/L bovine serum albumin (BSA), and incubated with primary antibody for 60-min. After that, a secondary antibody conjugated with fluorescein isothiocyanate (DAKO, Denmark) was added and the incubation was allowed to proceed for 60 min at 37°C. After washing, the coverslips were examined under a fluorescence microscope (Nikon Eclipse TE 2000-U, Kanagawa, Japan).

Chromosome preparation and G-banding analysis

The established tumor cells at 12th passage were subjected to chromosomal analysis. The cells were treated with 10 µmol/L Colchicine (Sigma, St. Louis, MO, USA) for 30 min and suspended in hypotonic solution, 0.075 mol/L KCl (Sigma, St. Louis, MO, USA), for 7 min, fixed in Carnoy's fixative and spreaded onto cold glass slides. The cells were stained with Giemsa and the representative chromosome sets were photographed for karyotype analysis. Interpretation of the karyotype was based on ISCN (1995). The modal chromosome number was determined from 20 cells.

Multicolour fluorescence in situ hybridization (mFISH) analysis

A duplicate slide, prepared from the same culture used for G-banding, was subjected to mFISH analysis according to the manufacturer's protocol (Metasystem, Germany). The 24 XCyte mFISH kit (Metasystem, Germany) with five fluorophors were used for hybridization, including FITC, Spectrum Orange, Texas Red, Cy5 and DEAC. Each chromosome (1-22, X and Y) was painted with different colors using various combinations of the fluorophores. After hybridization, the slides were evaluated under a fluorescence microscope (Axioimage, Zeiss, Germany), and the images were captured and analyzed using the Program Isis (Metasystem, Germany).

Tumor marker detection

A suspension of 10⁵ tumor cells was cultured in serum-free HAM's F12 medium for 24 h before the conditioned medium was collected and centrifuged at 1800 r/min for 10 min. The supernatant was then collected for detection of CA 19-9, CEA and AFP by using chemiluminescence detection system on an ELECHYS 2012 instrument (Roche, USA).

In vitro invasion assay

Cancer cell invasiveness was determined using transwell chamber (Costar, Cambridge, MA, USA) coated with 0.3 g/L matrigel (Collaborative Research Inc., Bedford, MA, USA). Approximately, 1 × 10⁵ cells (RMCCA-1, KKKU-100, KKKU-213 and HuCCA-1) in culture medium containing FBS were added into the upper compartment of the transwell, and incubated at 37°C in a humidified atmosphere containing 50 mL/L CO₂ for 6 h. The lower compartment contained culture medium plus FBS. The

filters were fixed with methanol and stained with 0.5% crystal violet in 25% methanol for 1 h, before rinsing with tap water several times. The cells on the upper surface of the filters were gently removed using cotton swabs and the cells that have invaded into the lower surface were counted under a microscope. The numbers of invaded cells in five random × 10 microscopic fields were counted and expressed as number of cells per well. The results shown represented mean ± SE of the number of invaded cells from three independent experiments, each carried out in triplicates.

In vitro motility assay

Motility assay was performed in a similar fashion to the invasion assay, except no matrigel coating was applied to the upper surface of the transwell filters. The numbers of migrating cells in five random × 10 microscopic fields were counted and expressed as number of cells per well. The results shown represented mean ± SE of the number of migrating cells from three independent experiments, each carried out in triplicates.

Gelatin zymography assay

Cells were starved by culturing in the medium without FBS for 24 h before collection of the conditioned medium. The conditioned medium was mixed with 5 × SDS-sample buffer before separation in a 120 g/L SDS-PAGE containing 1 mg/mL gelatin (Sigma, St. Louis, MO, USA). After electrophoresis at 200 V for 1 h, the gel was washed in a 25 mL/L Triton X-100 (Amersham, Piscataway, NJ, USA) solution twice. The gel was then incubated in buffer containing 50 mmol/L Tris-HCl (Amersham, Piscataway, NJ, USA), pH 7.5, 10 mmol/L CaCl₂ (Merck, Denmark), 1 mmol/L ZnCl₂ (Merck, Denmark), 10 mL/L Triton X-100 for 16-18 h, after which the gel was stained with 5 g/L Coomassie blue in 300 mL/L methanol and 100 mL/L acetic acid. After destaining, the clear band of gelatinolytic activity was documented for size determination using a Biorad GS700 gel scanner (Biorad, Hercules, CA, USA)^[9].

RESULTS

Primary cell culture

The human CCA tissue fragments adhered to the dish after plating for 6 h. After three weeks, a layer of epithelial cells appeared, from which contaminating spindle-shaped fibroblasts could be readily distinguished and separated from the epithelial tumor cells by differential trypsinization. After successive 16 passages, a homogeneous immortalized culture of tumor cells was established and was named RMCCA-1. The cells from passages 16th and 30th were then used for morphological analysis. These cells exhibited circular to spindle shape with many processes and ornamental fringes. The nucleus and cytoplasm appeared granulated (Figure 2).

Growth kinetics

RMCCA-1 cells were in lag phase until d 5, after which they entered a logarithmic growth phase. The doubling time determined from the slope of the growth curve was 48 h (Figure 3).

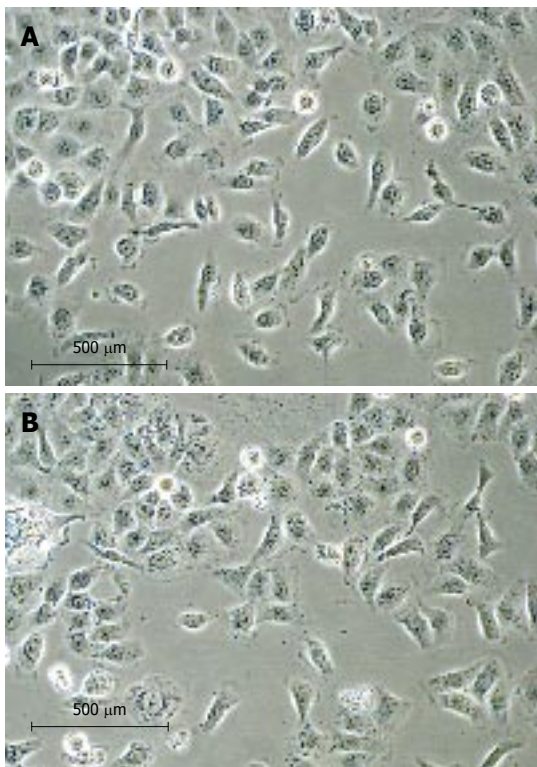


Figure 2 The RMCCA-1 culture under a phase contrast microscope at 20 × magnification. (A) at 16th passage; (B) at 30th passage. The RMCCA-1 cells exhibited circular to spindle shape with many processes and ornamental fringes. The nucleus and cytoplasm appeared granulated.

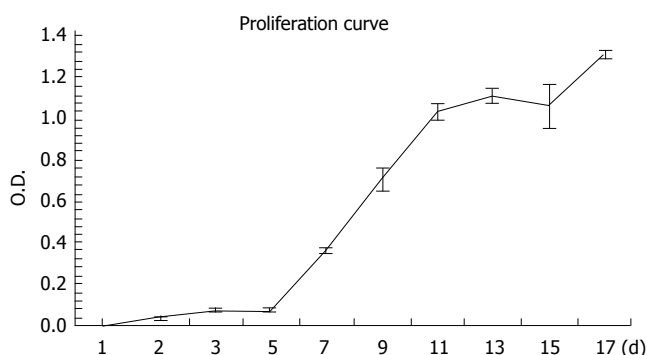


Figure 3 Growth kinetics of the RMCCA-1 cell line *in vitro* as analyzed by MTT assay. The tumor doubling time during the exponential phase of growth was 48 h. Each point represents mean \pm SE from 3 independent experiments, each performed in triplicates.

Immunofluorescence staining

All RMCCA-1 cells showed positive staining with the AE-1/AE-3 monoclonal antibody mixture (Figure 4B), which recognizes the human epidermal cytokeratins, the signature of epithelial cells that distinguishes them from fibroblasts. Furthermore, specific markers for the adenocarcinoma and transitional cell carcinomas, cytokeratin 7 (Figure 4A) and 19 (data not shown), were also positive.

Chromosome analysis

The G-banding analysis demonstrated aneuploidy karyotype with marked structural abnormalities of the

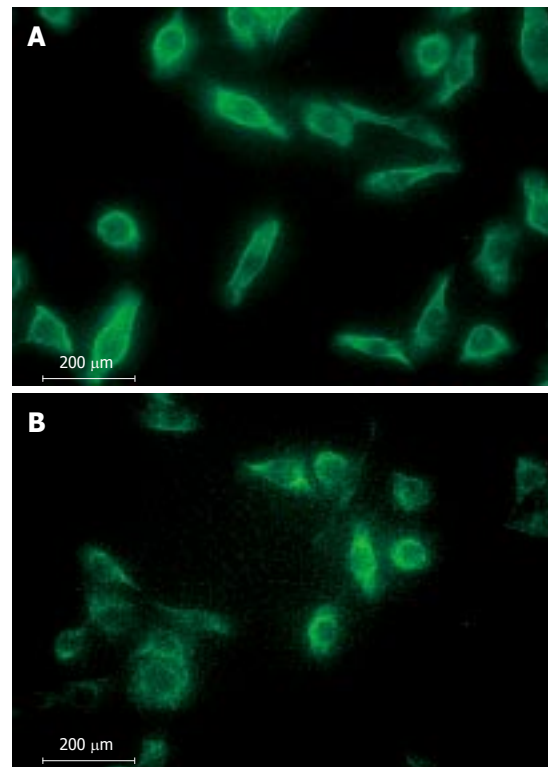


Figure 4 Immunofluorescence staining of RMCCA-1 cells with antibodies against (A) Cytokeratin 7; (B) AE-1/AE-3 at 40 × magnification.

chromosomes (Figure 5A and B). Although this cell line was established from a male patient, it lacks a Y chromosome and, instead, possesses two X chromosomes. The number of chromosomes ranges between 54 and 61, with a modal chromosome number of 59. The final karyotype was determined by consolidating the G-banding and mFISH results. Sixteen structural rearrangements were found, including 9 unbalanced translocations and 2 balanced translocations. In addition, the absence of Y chromosome in this cell line was also confirmed by both G-banding and mFISH techniques.

Tumor markers in spent media

The level of CA19-9 in the spent medium of RMCCA-1 cells (72.92 ± 4.35 U/mL) was similar to that of the patient's serum at admission (85.05 U/mL); both of which were about twice the maximum value of the normal range (39 U/mL, Table 1). The levels of CEA and AFP were normal (Table 1).

Invasiveness and motility

An important characteristic of metastatic cancer is the ability to migrate and invade the underlining basement membrane, the surrounding tissues and the blood vessels. We thus examine the invasiveness and motility of the RMCCA-1 cells, compared with those of three other established Cholangiocarcinoma cell lines from Thai patients (KKU-100, KKU-213 and HuCCA-1), using *in vitro* invasion and motility assay, respectively^[10]. RMCCA-1 exhibited relatively higher migration rate ($1,688 \pm 207$ cells/well) compared with the other 3 cell lines ($1,514 \pm 152$ cells/well, $1,123 \pm 163$ cells/well, 40 ± 17 cells/well

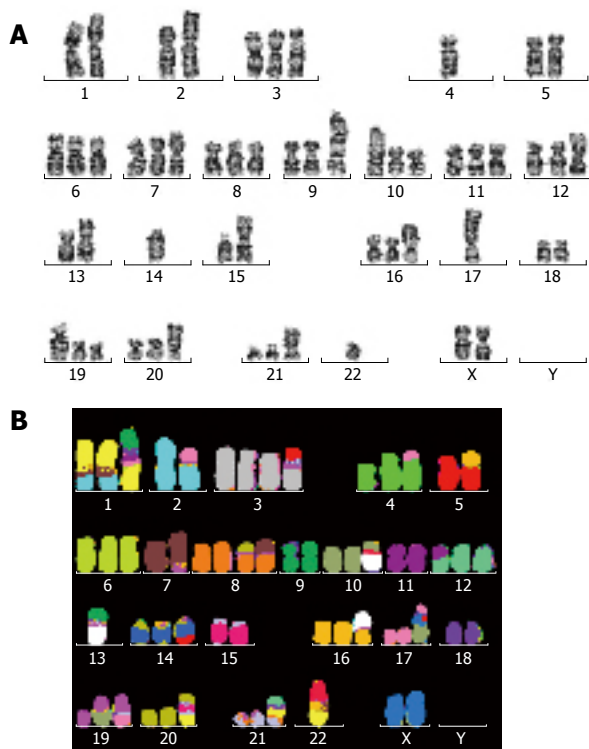


Figure 5 Representative karyotypes of RMCCA-1 cell line as assessed by (A) G-banding; (B) mFISH technique. The karyotype showed 46-61(3n)XX, -Y, der(1), t(1;2)(q31;q31), der(2)t(2;17)(q33;q12), der(3)t(3;15)(q21;q?), t(4;17)(p14;q?), der(7)t(7;15)(p22;q?), der(8)t(8;7)(p12;q?), der(10)t(10;13)(q21;q11), der(12)t(11;12)(q?,p11.2), der(13)t(13;9)(q11;q11), der(14)t(15;14)(q34;q32), der(16)t(13;16)(q11;p13.3), der(17)t(X;10;17)(p?,p?,p11.2), der(19)t(10;19)(q11.2;p13.3), der(19)t(17;19)(q?,q?), der(21)t(1;9;21)(?,q11), der(22)t(1;15;22)(?,?,?).

for KKU-213, KKU-100 and HuCCA-1, respectively). Surprisingly, the invasiveness of RMCCA-1 was significantly lower than those of KKU-213 and KKU-100 (181 ± 54 cells/well for RMCCA-1 vs 1021 ± 5 cells/well for KKU-213 and 765 ± 244 cells/well for KKU-100), while slightly higher than that of HuCCA-1 (38 ± 21 cells/well) (Figure 6).

Gelatin zymography

Most metastatic cells secrete proteinases to facilitate its invasion through tissue barriers. One of the most characterized families of tissue-degrading enzymes from cancer is the matrix-metalloproteinases (MMPs). Here we examined the ability to secrete the MMPs from RMCCA-1 cells by gelatin zymography. KKU-100 secretes a high level of Mr 72000 band previously shown to correspond to MMP-2 (Figure 7)^[11,12]. A Mr 72000 band is also present in FBS. Presumably, this band corresponds to the endogenous gelatinase activity present in the FBS (our unpublished observation). In contrary, the conditioned medium of RMCCA-1 lacks the activity (Figure 7).

DISCUSSION

Cholangiocarcinoma (CCA) is a cancer arising from the bile duct epithelium. It is a lethal disease of which little is known about its biology, pathogenesis and behavior. Up to present, there has been no effective early detection protocol or curative strategy for the disease; most patients

Table 1 Tumor marker levels in the spent media of RMCCA-1 and in the patient's serum at admission

	CEA (ng/mL)	AFP (U/mL)	CA 19-9 (U/mL)
¹ RMCCA-1	< 0.200	< 0.500	72.92 ± 4.35
Patient's serum	7.56	2.24	85.05
Normal serum	0.0-3.4	0.0-5.8	0.0-39

¹The results shown represented mean ± SE from three independent experiments.

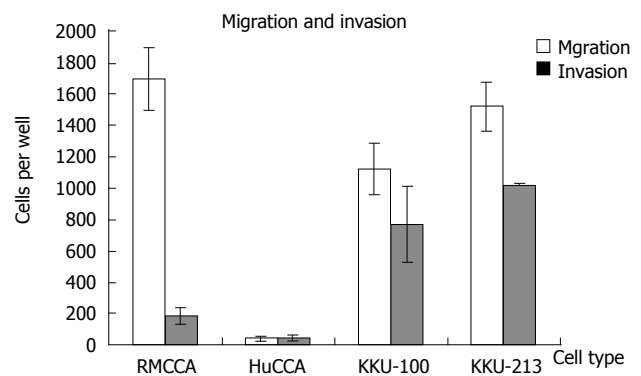


Figure 6 Invasion and migration rates of RMCCA-1 compared with KKU-213, KKU-100 and HuCCA-1 cell lines as determined by *in vitro* invasion and motility assay. Approximately 1×10^5 cells were seeded into the upper chamber of a transwell, and incubated for 6 h before the filter was fixed, stained, and the invading/migrating cells were counted under microscope.



Figure 7 MMPs activity as determined by gelatin zymography. An intense band at Mr 72000 was shown in the FBS (fetal bovine serum) and the conditioned medium of KKU-100, but not of the RMCCA-1 cells. The Mr 72000 band in KKU-100 has previously been shown to correspond to MMP-2 activity in many cell lines^[11,12].

seek curative treatment at advanced stage with poor prognosis. Therapeutic options for cholangiocarcinoma have been limited due to poor response to chemotherapy and radiation therapy. Surgery is perhaps the only effective cure although the 5-year survival after surgical treatment is less than 40%^[2,5]. Therefore, it is urgently required that the molecular markers for early detection are mapped and molecular targets for effective treatment are deciphered. One way to understand the nature of such disease is by studying the behavior of cell lines derived from the tumor *in vitro*. Here we describe the establishment and preliminary characterization of a cell line derived from a peripheral cholangiocarcinoma of a Thai patient which we have named RMCCA-1.

The population doubling time of this cell line was about 48 h, where those of other cholangiocarcinoma cell lines originated from Thai patients were 55 h for HuCCA1^[7] and 72 h for KKU100^[8]. Although we have not yet deciphered the mechanism of growth regulation for

RMCCA-1, growth regulation mediated by COX-2, PGE₂, EGFR and Akt had been demonstrated in a number of cholangiocarcinoma cell lines, including CCLP1, HuCCT1, SG231^[13].

Analysis of the spent medium from RMCCA-1 cells showed that these cells have retained some functional characteristics of the original tumor, including over expression of the tumor marker CA19-9. The levels of CA19-9 in the spent medium of RMCCA-1 cells (72.92 ± 4.35 U/mL) was similar to that of the patient's serum at admission (85.05 U/mL); both of which were approximately twice the maximum value of the normal range (39 U/mL, Table 1). CA19-9 was often detected in patients with malignant cholangiocarcinoma and pancreatic cancer^[14]. Many kinds of cancer cell lines also secrete CA19-9, including those derived from cholangiocarcinoma (TK)^[15], pancreatic carcinoma (SUIT-2)^[16] and colon cancer (SW1116)^[17].

Analysis by immunofluorescence staining using monoclonal antibodies to AE1 and AE3 showed positive staining for human cytokeratins, verifying the epithelial origin of the tumor. RMCCA-1 also stained positively with antibodies to cytokeratins 7 and 19, distinguishing the bile duct epithelial cells from the hepatocytes. Together, these data confirm that RMCCA-1 indeed derived from the epithelium of the bile duct.

Tumor metastasis involves a series of complex processes including dysregulation of cell adhesion, cell motility, and enzymatic proteolysis of basement membrane and extracellular matrix. The RMCCA-1 cells exhibit a high level of migration rate, contradicting the relatively low level of invasiveness and the absence of MMP activity (Figures 6 and 7). The low level of invasiveness in RMCCA-1 can, at least in part, be explained by the lack of MMP activity in this cell line. Unlike the RMCCA-1 cells, KKU-100 exhibits a high degree of invasiveness, correlating with a high level of MMPs activity (Figures 6 and 7). Karyotype analysis by G-banding revealed a complex pattern of chromosomal abnormalities. Most of the chromosomes were triplicates, suggesting that the cell line was originated from a triploid cell. The lost of Y chromosome was not unusual as similar finding has been shown in human sarcomatous cholangiocarcinoma (SCK) cells^[18]. Other aberrations involving Y chromosome have also been reported, including the translocation between Y chromosome and chromosome 1 as shown in a human cholangiocarcinoma cell line, PCI-SG231^[19].

With recent development of mFISH technique, it has become possible to identify and characterize complex chromosomal aberrations, previously unrevealed by G-banding analysis. This technique has allowed us to characterize the karyotype of RMCCA-1 in much greater detail with a high level of accuracy.

We have detected aberrations of chromosomes 1, 5, 7, and 12 in the RMCCA-1 cells, which were consistent with those of the other human cholangiocarcinoma cell lines including SCK, JCK, Cho-CK, Choi-CK, CC-SW-I, CC-LP-I, PCI: SG231, and RPMI-7451, and a rat cholangiocarcinoma cell line, CC-62^[18-21]. In contrast, we did not detect structural rearrangement of X chromosome and chromosome 6 in the RMCCA-1 cells, nor did we

detect the lost of chromosome 18, as found in SCK, JCK, Cho-CK, and Choi-CK^[18].

In conclusion, we successfully established a new cholangiocarcinoma cell line which we named RMCCA-1. We have also performed preliminary characterization of its growth characteristics, karyotype, secreted tumor markers and invasive properties. This cell line will be further used in our research towards understanding and combating against cholangiocarcinoma.

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Enhanced expression of interleukin-18 in serum and pancreas of patients with chronic pancreatitis

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Abstract

AIM: To investigate interleukin-18 (IL-18) in patients with chronic pancreatitis (CP).

METHODS: We studied 29 patients with CP and 30 healthy controls. Peripheral blood mononuclear cells (PBMC) were isolated and incubated with 50 mmol/L ethanol, lipopolysaccharide (LPS) (doses 25 g/L, 250 g/L, 2500 g/L) and both agents for 24 h. Levels of IL-18 in the supernatants, and levels of IL-18, IL-12, interferon (IFN)- γ and soluble CD14 in the serum were analysed by ELISA technique. Expression of IL-18 in PBMC was investigated by reverse-transcription (RT)-PCR. IL-18 protein levels in CP tissue and in normal pancreas were studied by ELISA technique. IL-18 levels in PBMC and pancreatic tissue were determined by Westernblot. Immunohistochemistry for pancreatic IL-18 expression was performed.

RESULTS: In patients, IL-18 serum levels were significantly enhanced by 76% (mean: 289.9 ± 167.7 ng/L) compared with controls (mean: 165.2 ± 43.6 ng/L; $P < 0.0005$). IL-12 levels were enhanced by 25% in patients (18.3 ± 7.3 ng/L) compared with controls (14.7 ± 6.8

ng/L, $P = 0.0576$) although not reaching the statistical significance. IFN- γ and soluble CD14 levels were not increased. *In vitro*, LPS stimulated significantly and dose-dependently IL-18 secretion from PBMC. Incubation with ethanol reduced LPS-stimulated IL-18 secretion by about 50%. The mRNA expression of IL-18 in PBMC and the response of PBMC to ethanol and LPS was similar in CP patients and controls. In PBMC, no significant differences in IL-18 protein levels were detected between patients and controls. IL-18 protein levels were increased in CP tissues compared to normal pancreatic tissues. IL-18 was expressed by pancreatic acinar cells and by infiltrating inflammatory cells within the pancreas.

CONCLUSION: IL-18 originates from the chronically inflamed pancreas and appears to be involved in the fibrotic destruction of the organ.

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Key words: Chronic pancreatitis; Cytokines; Interleukin-18; Pancreatic fibrosis

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INTRODUCTION

Chronic pancreatitis represents an inflammatory disease characterized by repeated attacks of acute pancreatitis, severe abdominal pain, progressive destruction of the pancreatic tissue with fibrous replacement of the parenchyma leading to both exocrine and endocrine insufficiency^[1]. In industrialized countries, excessive alcohol consumption is associated with the development of chronic pancreatitis in the majority of patients^[2,3]. The early stages of the human disease remain almost inaccessible to investigation. Recent genetic findings suggest that premature digestive enzyme activation with subsequent pancreatic autodigestion represents a dominant factor in the initiation of acute pancreatitis^[4,5]. These genetic studies also support a progressive link between repeated episodes

of acute pancreatitis and the development of chronic pancreatitis^[5]. However, the exact immune mechanisms underlying the progression of chronic pancreatitis remain unclear.

The identification and characterization of pancreatic stellate cells (PSC) provided deep insights into the development of pancreatic fibrosis^[6,7]. Activated PSC synthesize and secrete increased amounts of extracellular matrix proteins resulting in the fibrotic destruction of the pancreas^[6,7]. Recent *in vitro* studies have demonstrated that alcohol and its metabolite acetaldehyde^[8], oxidative stress^[8], growth factors such as platelet derived growth factor^[6], and the cytokine transforming growth factor (TGF)- β 1^[6] have the capacity to activate PSC during pancreatic injury. More recently, it was shown that PSC also responds to additional proinflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, IL-6, and antiinflammatory cytokines such as IL-10^[9].

During chronic pancreatitis, lymphocytes and mononuclear cells infiltrate the pancreas and contribute to the local progression of the disease through T-lymphocyte mediated cytotoxicity and production of cytokines^[10-14]. However, data regarding the role of cytokines in chronic pancreatitis remain limited. IL-18 represents a proinflammatory cytokine that plays an important role in the Th-1 response due to its ability to induce interferon (IFN)- γ production in T-cells and natural killer cells^[15,16]. IL-18 has been investigated in a variety of inflammatory and autoimmune human diseases^[17]. In previous studies, our group and others have demonstrated an upregulation of serum IL-18 levels in patients with acute pancreatitis^[18-20]. *In vitro* studies have shown that endotoxin induces IL-18 gene expression and secretion in human peripheral blood mononuclear cells (PBMC)^[21]. In a previous investigation, the serum levels and gene expression of IL-18 in PBMC of patients with alcoholic liver cirrhosis were significantly enhanced compared to healthy controls, and IL-18 levels correlated with plasma endotoxin levels^[22].

These data raise the possibility that IL-18 also participates in the immune mechanisms that result in the fibrotic destruction of the pancreas during chronic pancreatitis. Thus, the aim of the present study was to investigate this cytokine in patients with chronic pancreatitis. We determined the serum levels of IL-18, IFN, IL-12 and soluble CD14 in patients with chronic pancreatitis and healthy controls. We performed several *in vitro* studies with PBMC. We determined the protein expression of IL-18 in chronic pancreatitis tissue and in normal pancreas, and conducted immunohistochemical investigations in human pancreatic tissues.

MATERIALS AND METHODS

Patients

A total of 29 patients (22 males, 7 females; mean age 52 ± 11 years) with alcoholic and non-alcoholic chronic pancreatitis and 30 healthy controls with no history of alcohol abuse (10 males, 20 females; mean age 39 ± 11 year) were prospectively enrolled into the study at the University Hospital of Heidelberg at Mannheim,

Mannheim, Germany. The study was approved by the Ethics Committee of the Faculty of Clinical Medicine Mannheim, University of Heidelberg, Germany. Written informed consent was obtained from each participant.

Data on the history of both alcohol consumption and the clinical course of pancreatic disease were assessed by patient self-report and review of the medical records. In each participant, a detailed history of alcohol intake was established using different screening methods, including the Lübeck alcohol dependence and abuse screening test (LAST)^[23], the alcohol use disorders identification test (AUDIT)^[24], the lifetime drinking history (LDH)^[25], and a patient interview questioning ICD-10 criteria of chronic alcohol dependence^[26]. Alcoholic disease etiology was established with the presence of at least one of the following criteria: (1) patient self-report of alcohol abuse as cause of the disease, (2) patient self-report with a history of excessive alcohol intake of at least 80 g per day in males and 60 g per day in females for some years, or (3) smaller amounts of daily alcohol intake in combination with answers gained from the above mentioned screening methods for alcohol consumption that allowed the diagnosis of chronic alcohol abuse.

The diagnosis of chronic pancreatitis required the typical clinical features of chronic pancreatitis with or without recurrent episodes of acute pancreatitis, and was based on the determination of pancreatic exocrine and endocrine function, pancreatic imaging or histological tissue examination. Pancreatic imaging was performed either by endoscopic retrograde pancreatography (ERP), computed tomography (CT), magnetic resonance imaging (MRI), or endosonography. In each patient, abdominal ultrasound was performed. According to the definitions of an international workshop on chronic pancreatitis^[27], patients were further classified as suffering “definite” or “probable” chronic pancreatitis. Briefly, the classification of “definite” chronic pancreatitis required a typical clinical history of chronic pancreatitis and one or more of the following criteria: (1) calcifications in the pancreas, (2) moderate to marked ductal lesions, (3) marked exocrine insufficiency, and (4) typical histology of an adequate surgical specimen^[27]. For the diagnosis of “probable” chronic pancreatitis, one or more of the following criteria were present in addition to the typical clinical features: (1) mild ductal alterations, (2) recurrent or persistent pseudocysts, (3) pathological secretin test, and (4) endocrine insufficiency^[27].

In all patients, an episode of acute pancreatitis at the time of recruitment or within the last two months before inclusion into the study was excluded. Additional exclusion criteria were infections with fever or leukocytosis, liver cirrhosis and surgical or endoscopic interventions within 2 mo before examination.

Routine laboratory parameters

Routine laboratory parameters were determined in serum samples taken at the same time as samples for cytokine and endotoxin measurement. These parameters included serum concentrations of amylase, lipase, C-reactive protein (CRP), creatinine, and total white blood cell count. Endocrine pancreatic insufficiency was determined by the presence

of diabetes mellitus requiring antidiabetic treatment or records of an abnormal oral glucose tolerance test. Exocrine pancreatic insufficiency was defined by diarrhea, steatorrhea or maldigestion that was markedly reduced by enzyme supplementation. In some patients, pancreatic exocrine function was determined by using one or more of the following commercially available tests according to the manufacturer's recommendations: measurement of fecal chymotrypsin by a colorimetric method (Chymo, Boehringer, Germany), determination of fecal fat excretion by infrared reflection method (Esetek Analyser Fenir 8820, TSZ Stimotron AG, Wettengel-Launsbach, Germany), measurement with the Pankreolauryl test N (Temmler Pharma GmbH, Marburg, Germany) or application of the pancreozymin-secretin-test (Sekretolin Diagnostikum, Hoechst AG, Germany or Takus, Pharmacia GmbH, Erlangen, Germany).

PBMC isolation and incubation with ethanol and endotoxin

Peripheral venous blood was collected from patients and healthy controls into sterile, pyrogen-free disposable syringes with endotoxin-free heparin (10 ku/L). As reported previously^[22], PBMC was separated by standard density gradient centrifugation (Ficoll-Paque method) and adjusted to 3×10^9 cells/L in RPMI 1640 supplemented with 100 mL/L heat inactivated fetal bovine serum. PBMC were incubated with or without lipopolysaccharide (LPS) (doses 25 g/L, 250 g/L, 2500 g/L), in the absence or presence of 50 mol/L ethanol for 24 h. Cells were spun down, and PBMC supernatants were stored at -20°C until measurement of cytokine levels. For RNA extraction, cells were stored at -80°C in 4 mol/L GTC extraction buffer.

Immunoassay for IL-18, IFN, IL-12, soluble CD14

Concentrations of IL-18 in the serum, supernatants of PBMC and pancreatic tissues were determined by a specific sandwich enzyme linked immunoassay (ELISA; Fujisaki Institute, Hayashibara Biochemical Laboratories, Inc., Okayama, Japan) with minor modifications as described previously^[22]. Serum concentrations of IFN were determined by a specific ELISA using two monoclonal antibodies as described previously^[28]. Serum concentrations of IL-12 and soluble CD14 were also determined by ELISA technique as described previously^[28].

RNA isolation and IL-18 RT-PCR analysis in PBMC

RNA extraction from PBMC was performed using acid phenol-chloroform extraction^[22]. The RNA concentration was quantified spectrophotometrically. Complementary DNA of PBMC was obtained by reverse-transcription (RT) using 1 µg RNA and oligo d(T) primers. PCR for IL-18 and the housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed as described previously^[22]. Densitometric assessment of the PCR products was performed using EASY Plus 3.2 software (Herolab, Wiesloch, Germany). Semiquantitative PCR results were obtained by grading a ratio between the densitometry results of IL-18 and GAPDH.

Endotoxin assay

Plasma endotoxin levels were determined using an

automated kinetic turbidimetric limulus amoebocyte lysate microtiter test as described previously^[22].

Pancreatic tissue samples

Pancreatic tissue samples were obtained from 8 patients with chronic pancreatitis and from 2 individuals without pancreatic disease. Two of these patients with chronic pancreatitis were obtained from the present investigation and had later to be operated due to the development of a benign pancreatic mass and intractable pancreatic pain. The remaining 6 patients with chronic pancreatitis and the 2 individuals without pancreatic disease were obtained from clinical routine interventions. In 4 of these patients with chronic pancreatitis, surgery was necessary due to the development of pancreatic cancer.

Human pancreatic tissue and cell lysates of PBMC were homogenized in 5 mmol/L Hepes, pH 7.0, 280 mmol/L mannitol, 10 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L benzamidine, 20 mg/L trypsin inhibitor, 1 µmol/L leupeptin, and 0.2 mmol/L PMSF and boiled in electrophoresis sample buffer. Proteins were electrophoretically separated on SDS-125 g/L polyacrylamide gels. Electrotransfer to nitrocellulose membranes was done as described previously^[29]. After staining with 2 g/L Ponceau S to check the efficiency of the transfer, free binding sites of the membrane were blocked with 10 g/L bovine serum albumin in Tris-buffered saline (10 mmol/L Tris-HCl, pH 8.0, and 150 mmol/L NaCl) for 60 min, followed by 75 min incubation with anti-IL-18 antibody (NatuTec, Frankfurt, Germany) diluted in Tris-buffered saline plus 2 mL/L Tween 20. Bound antibodies were visualized with secondary antibodies conjugated to horse radish peroxidase using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Freiburg, Germany) and autoradiography film (Fujifilm Super HR-E 30, Fuji photo film, Düsseldorf, Germany).

Immunohistochemistry

Frozen tissue specimen were cut in 10 µm cryostat sections, transferred on glass slides and air-dried overnight. Immunohistochemistry was performed according to the streptavidin-biotin method. Sections were washed in tris-buffered saline (TBS) and incubated with 30 g/L bovine serum albumine (BSA) for 10 min at room temperature to block non-specific antibody reactions. The sections were incubated overnight at 4°C with the primary antibody at a 1:5000 dilution (anti-human IL-18 polyclonal antibody, NatuTec, Frankfurt am Main, Germany). The slides were then rinsed repeatedly with TBS and were incubated with a biotin-streptavidin-conjugated secondary antibody (goat anti-rabbit immunoglobulin-specific antibody, Jackson Immuno Research, West Grove, USA) for 30 min at room temperature. The slides were washed again with TBS and were treated with a streptavidin-alkaline-phosphatase complex. Liquid diaminobenzidine was added as chromogen, and counterstaining was performed with hematoxylin. Controls were performed by using mouse serum (Sigma, Saint Louis, USA) as primary antibody and by administration of recombinant human IL-18 (MoBiTec, Göttingen, Germany) in excess to the primary antibody

before incubation. Light microscopical investigations were performed using a Zeiss Axioskop microscope.

Statistical analysis

We used the *t* test, if necessary with the Welch correction for unequal variances, for analysis of IL-18, IFN, IL-12 and soluble CD14 levels between patients and controls. These data are expressed as mean \pm SD. *P* < 0.05 was considered significant. We used paired *t* tests for the comparison of IL-18 secretion from PBMC. The Bonferroni-Holm correction was applied to adjust for calculating three tests at a time to compare the IL-18 secretion from PBMC after different stimulations.

RESULTS

Clinical characteristics

Alcoholic chronic pancreatitis was diagnosed in 23 patients, and non-alcoholic chronic pancreatitis was found in 6 patients (Table 1). According to the recommendations of a workshop on chronic pancreatitis, 21 patients were classified with “definite” chronic pancreatitis, and 8 patients were classified with “probable” chronic pancreatitis (Table 1). An episode of acute pancreatitis was excluded in all patients by physical examination, abdominal ultrasound and determination of routine laboratory parameters (Table 2).

Serum levels of IL-18, IL-12 and IFN in CP patients

The fasted serum IL-18 levels were significantly enhanced by 76% in 29 patients with chronic pancreatitis (mean \pm SD: 289.9 \pm 167.7 ng/L) compared to healthy controls (*n* = 30; 165.2 \pm 43.6 ng/L; Welch's *T* test, *P* < 0.0005). The IL-12 levels were enhanced by 25% in patients (*n* = 27 due to lack of sample in 2 patients; mean \pm SD: 18.3 \pm 7.3 ng/L) compared to controls (*n* = 30, 14.7 \pm 6.8 ng/L). We observed a trend towards a statistically significant difference between patients and control subjects (*T* test, *P* = 0.0576). Serum IFN levels were not increased in patients (*n* = 25 due to lack of sample in 4 patients; 28 \pm 15.4 ng/L) compared to controls (*n* = 24 due to lack of sample in 6 controls; 34.4 \pm 18.0 ng/L; *T* test, *P* = 0.18).

Serum soluble CD14 and plasma endotoxin levels in CP patients

Serum levels of soluble CD14 were similar in patients (*n* = 16, mean \pm SD: 2723.3 \pm 649.2 ng/L) and healthy controls (*n* = 26, mean \pm SD: 2630.6 \pm 480.3 ng/L; Welch's *T*-test: *P* = 0.63). Endotoxemia was not detectable in patients (*n* = 29) and controls (*n* = 30).

Expression of IL-18 mRNA in PBMC of CP patients

We investigated the IL-18 mRNA expression in PBMC of patients with chronic pancreatitis by RT-PCR to determine if the enhanced serum IL-18 levels in chronic pancreatitis represent a result of an increased gene expression in PBMC. No significant difference was found between the mRNA expression of IL-18 in patients with chronic pancreatitis (*n* = 5 patients with highest IL-18 serum levels) in comparison to healthy controls (*n* = 5 control individuals with lowest IL-18 serum levels) (Figure 1). The semiquan-

Table 1 Clinical characteristics of patients with chronic pancreatitis and healthy controls

Clinical characteristics	Patients	Controls
<i>n</i>	29	30
Age (yr)	52 \pm 11	39 \pm 11
Sex (Male/Female)	22/7	10/20
Alcoholic chronic pancreatitis	23/29	NA ¹
Non-alcoholic chronic pancreatitis	6/29	NA ¹
Definite chronic pancreatitis	21/29	NA ¹
Probable chronic pancreatitis	8/29	NA ¹
Exocrine insufficiency	16/29	NA ¹
Endocrine insufficiency	11/29	NA ¹

¹Not applicable.

Table 2 Routine laboratory parameters in patients with chronic pancreatitis and healthy controls (mean \pm SD)

Parameter	Normal range	Patients	Controls
White cell blood count	3.6-11.0 $\times 10^9$ /L	7.0 \pm 1.9	6.0 \pm 1.2
C-reactive protein	< 5 mg/L	6.4 \pm 7.7	3.2 \pm 0.9
Creatinine	6-11 mg/L	0.9 \pm 0.3	0.9 \pm 0.2
Amylase	8.4-31.7 U/L	33.0 \pm 22.6	33.0 \pm 17.4
Lipase	< 190 U/L	182.0 \pm 98.0	161.0 \pm 64.0

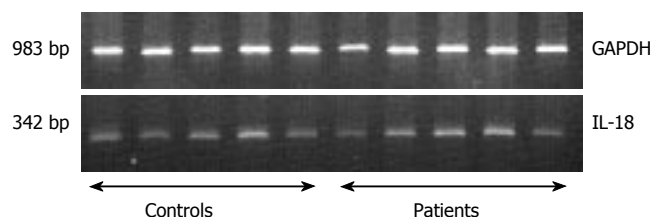


Figure 1 Analysis of RT-PCR amplification of IL-18 in PBMC of CP patients.

titative analysis of the ratio between the densitometric results from the PCR-products of IL-18 and GAPDH did not reveal a significant difference. The ratio for patients with chronic pancreatitis and healthy controls was 0.438 \pm 0.074 and 0.410 \pm 0.082, respectively.

IL-18 protein levels in pancreatic tissues

IL-18 protein levels in pancreatic tissues were determined by ELISA technique in two patients with chronic pancreatitis, and in two individuals without pancreatic disease. The protein levels of IL-18 in chronic pancreatitis tissue (*n* = 2, 50.5 ng/L and 86.1 ng/L) were enhanced compared to pancreatic tissue from individuals without pancreatic disease (*n* = 2, 33.6 ng/L and 25.1 ng/L). In these patients and control subjects, IL-18 protein levels in the pancreas were also determined by Westernblot (Figure 2A). The IL-18 protein levels were again enhanced in patients with chronic pancreatitis compared to the control individuals (Figure 2A).

IL-18 protein levels in cell lysates of PBMC

IL-18 protein levels were determined in cell lysates of PBMC by Westernblot investigations (Figure 2B). We

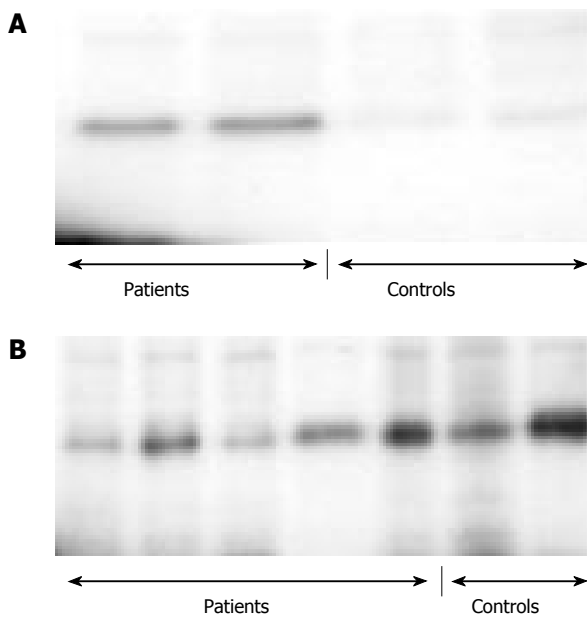


Figure 2 IL-18 in cell lysates of PBMC and in pancreatic tissue of CP patients. **A:** In the pancreatic tissue; **B:** In cell lysates of PBMC.

studied PBMC cell lysates from 5 patients with chronic pancreatitis and from 2 control individuals to determine if the elevated levels of IL-18 in patients with chronic pancreatitis result from an increased expression of IL-18 in PBMC. We confirmed IL-18 protein expression in the cell lysates of both patients and controls. However, we did not detect significant differences in IL-18 protein expression between cell lysates from patients and controls (Figure 2B).

IL-18 levels in the supernatants from PBMC after incubation with ethanol and LPS

We investigated the IL-18 levels in the supernatants of PBMC from all patients ($n = 29$) and all controls ($n = 30$). We stimulated the PBMC with ethanol and LPS to detect a possible influence of these agents on IL-18 secretion from PBMC and to reveal possible differences between PBMC from patients and control subjects. These *in vitro* studies demonstrated that the basal secretion of IL-18 from PBMC was similar in patients and control individuals.

The stimulation of PBMC with LPS (25 g/L, 250 g/L and 2500 g/L) and the stimulation with LPS (25 g/L, 250 g/L and 2500 g/L) together with ethanol (50 mmol/L) resulted in a dose related and statistically significant enhancement of IL-18 secretion from PBMC of patients and controls (T tests; $P < 0.0007$) (Figure 3A and B). However, we did not detect significant differences in the secretion patterns of IL-18 between PBMC from patients and control individuals.

The incubation with ethanol alone for 24 h did not affect basal IL-18 secretion, but ethanol significantly reduced LPS-stimulated IL-18 secretion by about 50% compared to LPS stimulation alone (T tests; $P < 0.0001$) (Figure 3A and B).

The application of the Bonferroni-Holm correction did not change the significance of previously obtained P values.

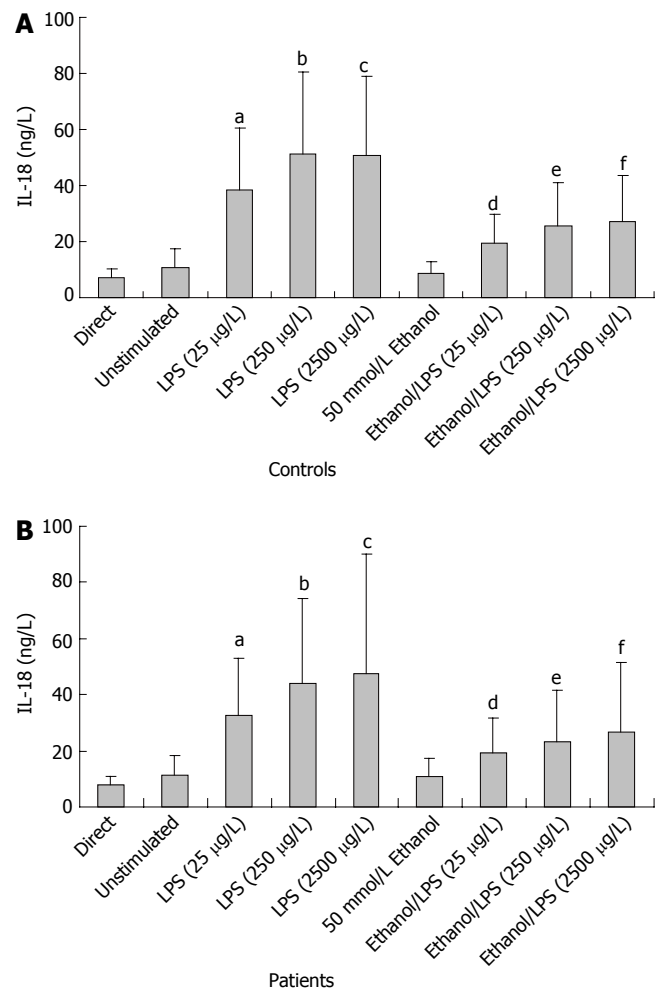


Figure 3 *In vitro* IL-18 secretion from PBMC of CP patients. ^a $P < 0.0007$ 25 µg/L LPS vs unstimulated; ^b $P < 0.0007$ 250 µg/L LPS vs unstimulated; ^c $P < 0.0007$ 2500 µg/L LPS vs unstimulated; ^d $P < 0.0001$ Ethanol/25 µg/L LPS vs 25 µg/L LPS; ^e $P < 0.0001$ Ethanol/250 µg/L LPS vs 250 µg/L LPS; ^f $P < 0.0001$ Ethanol/2500 µg/L LPS vs 2500 µg/L LPS.

Immunohistochemistry

We performed immunohistochemical investigations for IL-18 in pancreatic tissues from 6 patients with chronic pancreatitis. In 4 of these patients with chronic pancreatitis, invasive ductal adenocarcinoma of the pancreas had developed and was the reason for pancreatic surgery. In these patients, we investigated the chronic pancreatitis tissue which was free of pancreatic carcinoma tissues. The immunohistochemical analysis of the chronic pancreatitis tissue revealed that clusters of infiltrating mononuclear cells were stained positive for IL-18 by using anti-IL-18 antibody (Figure 4A). In chronic pancreatitis tissue, clusters of pancreatic acinar cells were also stained positive for the expression of IL-18 (Figure 4B). Thus, IL-18 appears to be expressed in both infiltrating inflammatory cells and pancreatic acinar cells during chronic pancreatitis. In addition, we studied sections from pancreatic carcinoma tissue, and detected positive staining for anti-IL-18 antibody in pancreatic carcinoma cells as well. In general, staining for IL-18 was more pronounced in the samples with pancreatic carcinoma than in the samples from patients with chronic pancreatitis without pancreatic carcinoma. Control investigations with mouse serum and with the IL-18-

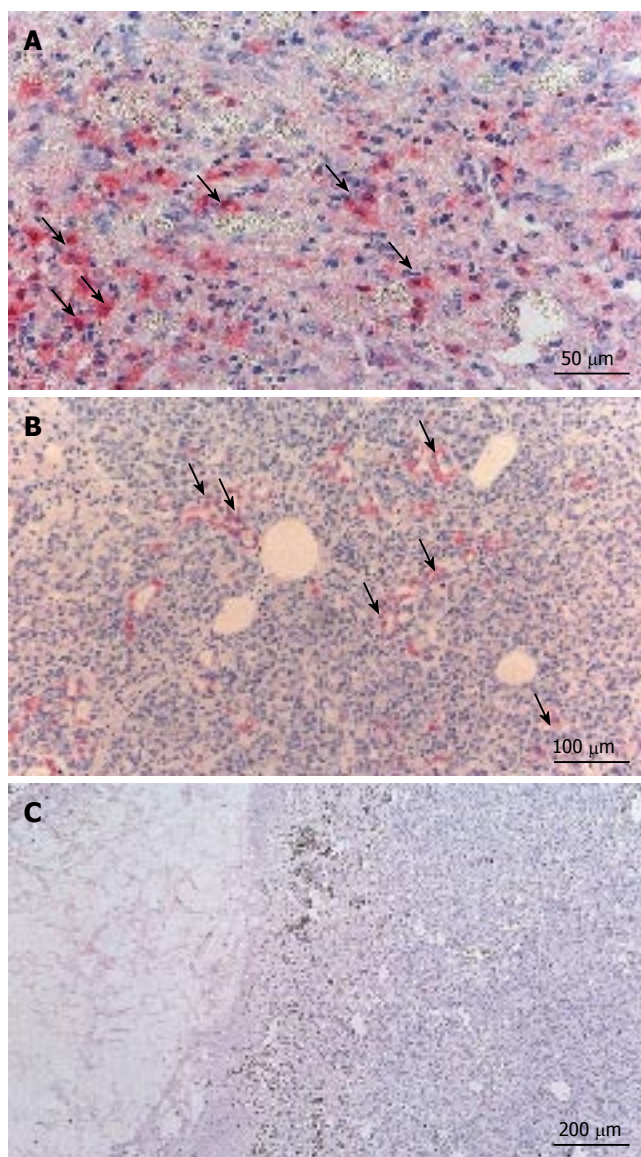


Figure 4 IL-18 expression in pancreatic tissue of CP patients. **A:** Clusters of infiltrating mononuclear cells stained positive; **B:** Clusters of pancreatic acinar cells also stained positive for the expression of IL-18; **C:** No positive staining for IL-18 in the negative control (mouse serum with IL-18 antigen-antibody mixture).

antigen-antibody-mixture revealed no positive staining for IL-18 and confirmed the accuracy of the method (Figure 4C).

DISCUSSION

The major findings of the present investigation are: (1) the fasted serum levels of IL-18 are significantly enhanced by 76% in patients with chronic pancreatitis compared to healthy control individuals; (2) the mRNA expression of IL-18, the protein levels of IL-18, and the *in vitro* secretion of IL-18 after stimulation with ethanol and endotoxin is similar in PBMC from patients with chronic pancreatitis and from healthy control subjects; (3) *in vitro*, ethanol significantly reduces endotoxin-stimulated IL-18 secretion by about 50%; (4) the IL-18 protein levels in chronic pancreatitis tissue are increased; and (5) IL-18 is expressed in pancreatic acinar cells and infiltrating inflammatory cells

within the pancreas.

These results suggest that PBMC are not the main source of the enhanced serum levels of IL-18 in patients with chronic pancreatitis. It rather appears that IL-18 originates from the chronically inflamed pancreas. Indeed, there is increasing evidence that immunological mechanisms play an important role in the development and progression of chronic pancreatitis^[9-13,30,31]. Cytokines, growth factors and other immunological mediators are produced by resident cells and recruited cells within the chronically inflamed pancreas and contribute to pancreatic fibrosis^[14]. In addition, pancreatic acinar cells also produce, release and respond to cytokines^[32]. Thus, we assume that IL-18 participates in the destruction of the pancreas during chronic pancreatitis.

Alcohol consumption represents an important risk factor for the development of chronic pancreatitis. Chronic alcohol consumption leads to an increased gut permeability with subsequent endotoxemia, and endotoxin has been reported as a mediator of alcohol induced liver damage^[33]. Studies in ethanol-fed rats also suggest a role of endotoxin in the development of pancreatic injury^[34]. Endotoxin strongly induced IL-18 gene expression in PBMC, and the response to endotoxin was mainly regulated by the expression of CD14^[21]. In our study, we did not detect endotoxin in the plasma of patients with chronic pancreatitis, and the soluble CD14 levels were similar in patients and control individuals. However, it has to be stressed that the present study was not designed to investigate the full impact of endotoxin on the development of pancreatic damage. Enhanced endotoxin plasma levels may only be detectable for short periods of time during acute ethanol consumption in humans, whereas the patients in our study had not consumed alcohol on the day of the investigation. In addition, not all patients with alcoholic chronic pancreatitis were still abusing alcohol at the time of recruitment into the study. Therefore, it appears that the enhanced blood levels of IL-18 do not result from endotoxin-mediated mechanisms.

Interestingly, our *in vitro* studies showed that the incubation of PBMC with ethanol decreased the endotoxin-stimulated secretion of IL-18 by PBMC thereby suggesting that ethanol consumption modulates the IL-18 expression in the pancreas. However, the role of IL-18 in alcoholic pancreatitis remains speculative since only limited data are available regarding the probably pleiotropic immunological function of IL-18 in chronic pancreatitis.

IL-18 participates both in Th1 and Th2 immune responses^[16]. IL-18 induces the production of cytokines such as TNF- α and IL-1 and enhances the production of further chemokines such as IL-8 and MCP-1^[35] that are increased in chronic pancreatitis tissue^[10,31,36]. Of note, IL-8 plays a major role in the recruitment of infiltrating neutrophils to the site of inflammation^[37]. The simultaneous presence of IL-12 and IL-18 results in a marked production of nitric oxide and reactive oxygen intermediates in macrophages and neutrophils^[38] which may also facilitate premature intrapancreatic trypsin activation and pancreatic autodigestion^[39]. The predominantly antiinflammatory cytokine IL-10 inhibits the synthesis of

proinflammatory cytokines such as TNF- α , IL-1, IL-6 and IL-8 and may play a dominant role in protecting the pancreas during pancreatic inflammation^[40,41]. There was only minimal induction of the antiinflammatory cytokines IL-1 receptor and IL-10 through IL-18^[35], and IL-10 failed to inhibit IL-18 production in response to inflammatory stimuli^[42]. Therefore, IL-18 may escape the influence of IL-10 during pancreatic inflammation.

Finally, among its pleiotropic effects, IL-18 strongly induces IFN- γ production^[15,16]. Increased levels of IFN were reported in chronic pancreatitis tissue from humans and animals^[13,43,44]. Interestingly, the fibrosis in chronic pancreatitis seems to be driven by TGF- β 1^[45], and IFN- γ and TGF- β 1 are linked by an antagonistic relationship^[46,47]. INF- γ possesses several antifibrotic characteristics, and IFN- γ has already been used therapeutically in animals and humans with fibrotic diseases^[47-49]. The recruitment and activation of infiltrating cells may depend on the local production of inflammatory mediators such as TGF- β 1 and IFN- γ ^[11,31]. Recent *in vitro* studies have demonstrated that TGF- β 1 strongly suppressed the production of IFN- γ that had been induced by costimulation with IL-18 and phytohaemagglutinin, a strong stimulator of IFN- γ synthesis, or by costimulation with IL-18 and IL-12^[50]. However, the role of IFN- γ in chronic pancreatitis is not completely clarified, and further studies are required to reveal the role of IL-18 during pancreatic fibrosis.

In conclusion, PSC are activated during pancreatic injury, and these cells produce and release increased amounts of extracellular matrix proteins thereby leading to the fibrotic destruction of the pancreas^[6,7]. Pancreatic stellate cells are activated and regulated on exposure to various proinflammatory cytokines^[9]. Thus, future studies should address the role of IL-18 in the context of PSC activation and regulation.

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Strong prognostic value of nodal and bone marrow micro-involvement in patients with pancreatic ductal carcinoma receiving no adjuvant chemotherapy

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Abstract

AIM: To study the prognostic value of adjuvant chemotherapy in patients with pancreatic, ductal adenocarcinoma.

METHODS: Lymph nodes from 106 patients with resectable pancreatic ductal adenocarcinoma were systematically sampled. A total of 318 lymph nodes classified histopathologically as tumor-free were examined using sensitive immunohistochemical assays. Forty-three (41%) of the 106 patients were staged as pT_{1/2}, 63 (59%) as pT_{3/4}, 51 (48%) as pN₀, and 55 (52%) as pN₁. The study population included 59 (56%) patients exhibiting G_{1/2}, and 47 (44%) patients with G₃ tumors. Patients received no adjuvant chemo- or radiation therapy and were followed up for a median of 12 (range: 3.5 to 139) mo.

RESULTS: Immunostaining with Ber-EP4 revealed nodal microinvolvement in lymph nodes classified as "tumor free" by conventional histopathology in 73 (69%) out of the 106 patients. Twenty-nine (57%)

of 51 patients staged histopathologically as pN₀ had nodal microinvolvement. The five-year survival probability for pN₀-patients was 54% for those without nodal microinvolvement and 0% for those with nodal microinvolvement. Cox-regression modeling revealed the independent prognostic effect of nodal microinvolvement on recurrence-free (relative risk 2.92, *P* = 0.005) and overall (relative risk 2.49, *P* = 0.009) survival.

CONCLUSION: The study reveals strong and independent prognostic significance of nodal microinvolvement in patients with pancreatic ductal adenocarcinoma who have received no adjuvant therapy. The addition of immunohistochemical findings to histopathology reports may help to improve risk stratification of patients with pancreatic cancer.

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Key words: Pancreatic ductal adenocarcinoma; Nodal microinvolvement; Micrometastases

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INTRODUCTION

Pancreatic adenocarcinoma is the fifth leading cause of death among all malignancies^[1], leading to approximately 40 000 deaths each year in Europe^[2]. Reported probabilities of five-year survival after curative surgery are still below 10 percent^[3]. Stage, grade and resection margin status are currently accepted as the most accurate pathologic variables predicting survival^[4-10]. Pathologic staging only insufficiently reflects the individual risk to develop tumor recurrence which is even high in early tumor stages. Thus, effort continues to identify new prognosticators of tumor

relapse that indicate the need of adjuvant therapy.

Occult residual tumor disease is suggested when either bone marrow or lymph nodes from which tumor relapse may originate are affected by micrometastatic lesions undetectable by conventional histopathology^[11]. The clinical significance of antibodies against tumor-associated targets both in lymph nodes^[12-15] and in bone marrow^[15,16] is still controversial^[16-26]. Various monoclonal antibodies are in use for micrometastatic detection, thus contributing to the incongruity of data and validity of results. These assays have been rarely used in patients with pancreatic carcinoma^[27-31]. Recently, our group showed that immunohistochemical staining with the monoclonal antibody Ber-EP4 is a sensitive and specific method for detecting isolated or clusters of tumor cells in lymph nodes from patients with lung^[13], esophageal^[25], or pancreatic carcinomas^[28]. Ber-EP4 is an antibody against two glycopolypeptides of 34 and 49 kD on the surface and in the cytoplasm of all epithelial cells (except parietal cells, hepatocytes, and the superficial layers of squamous epithelium).

The present study was to increase our knowledge gained in the previous studies on lymph node micrometastasis^[25,28,31,32]. In non-small cell lung carcinoma^[32], the risk to develop tumor relapse in pN1 patients is overall greater than in pN0 patients. However, we have shown that further risk stratification for patients with histopathological involvement may be performed according to their immunohistochemical status. Therefore, here we have extended our previously published study on lymph node-negative patients with pancreatic cancer^[31] with patients staged as pN1 through conventional histology as well as the bone marrow data from those patients that gave us written consent. The primary aim of this study was to assess the role of immunohistochemically detectable micrometastases in lymph nodes of an unselected group of patients with "curatively" resected pancreatic ductal adenocarcinoma. The secondary aim was to assess whether lymph node microinvolvement is correlated to bone marrow micrometastasis and which of these two sites is a better indicator for tumor cell dissemination in pancreatic cancer.

MATERIALS AND METHODS

Patients and study design

The local ethical committee of Hamburg approved this study. Informed consent was obtained from all patients before inclusion in the study. Tumor samples, lymph nodes, and bone marrow aspirates of the upper iliac crest were collected from 487 patients with pancreatic and periampullary malignancies. Out of these patients, 171 (35%) had carcinomas of the papilla of Vater, 47 (10%) exhibited carcinoma of the distal common bile duct, and 269 (55%) had pancreatic carcinoma. Out of these 269 patients, 49 (18%) had neuroendocrine tumors and 220 (82%) had true pancreatic carcinoma.

Our study population included 106 patients with resectable pancreatic ductal adenocarcinoma who had undergone curative surgery and had given informed consent for immunohistochemical analysis of lymph

nodes. Patients with cystic malignancies (IPMN, cystadenocarcinoma), acinar cell and squamous cell carcinomas were not considered for this study. The most frequent surgical procedure was pancreatoduodenectomy. Lymph node dissection was performed as previously described by Pedrazzoli *et al*^[33]. A total of 1643 lymph nodes were removed with a median number of 16 (range 7 to 38) lymph nodes per patient. Among all histopathologically negative lymph nodes, 318 were selected in a representative fashion as described most recently for subsequent immunohistochemical screening^[31]. Tumor stage and grade were classified according to the 6th edition of the tumor-node-metastasis classification (TNM) of the International Union against Cancer^[34] by investigators unaware of the immunohistochemical findings.

Follow-up evaluations at three-month intervals included a physical examination, abdominal ultrasonography, computed tomography of the abdomen and studies of tumor markers, i.e. carcinoembryonic antigen and CA 19-9. Out of all 106 patients studied, the vital status in 89 patients could be determined at the end of the study. Seventeen patients were excluded from the survival analysis because they were either censored or died within 90 d after surgery. From 3 patients, only information about the date of death but not of recurrence was available

Tissue preparation and immunohistochemical analysis

Lymph nodes were divided into two parts, one for conventional histopathology, the other was snap-frozen in liquid nitrogen within three hours after their removal and stored at -80°C until use. Only histopathologically "tumor-free" lymph nodes were screened by immunohistochemistry with the anti-epithelial-cell monoclonal antibody Ber-EP4 (IgG1; Dako, Hamburg, Germany) as described previously^[14]. Ber-EP4 is an antibody against two glycopolypeptides of 34 and 49 kD on the surface and in the cytoplasm of all epithelial cells (except parietal cells, hepatocytes, and the superficial layers of squamous epithelium). The antibody does not react with mesenchymal tissue, including lymphoid tissue^[15,23].

Cryostat sections (5 to 6 µm thick) were cut at three different levels in each node and transferred onto glass slides treated with 3-triethoxysilylpropylamin (Merck, Darmstadt, Germany). One section of the sample obtained at each level was stained by the alkaline phosphatase-antialkaline phosphatase technique combined with the new fuchsin stain (Sena, Heidelberg, Germany) for the visualization reaction^[23].

In 16 control patients with nonepithelial tumors or inflammatory diseases, lymph nodes were consistently stained negative. Sections of normal colon served as positive staining controls and isotype-matched, irrelevant murine monoclonal antibodies served as negative controls (purified immunoglobulin mouse myeloma protein for IgG1; Sigma, Deisenhofen, Germany).

The slides were evaluated in a blinded fashion by two observers working independently (D.B., J.T.K.). Minimal tumor cell involvement in a lymph node that was considered to be tumor-free by conventional histopathological staining was defined as the presence of

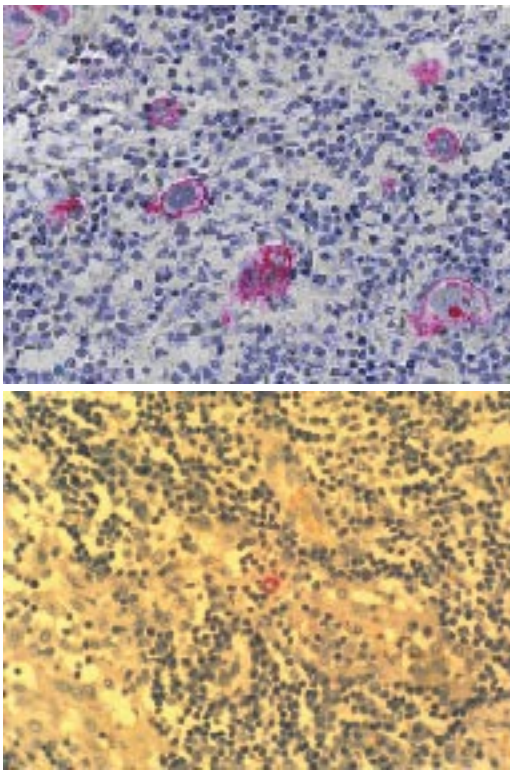


Figure 1 Immunohistochemically detectable nodal microinvolvement with monoclonal antibody Ber EP4 ($\times 400$).

one to ten positive cells in the body of the node (Figure 1). If more than 10 cells were detected (2 lymph nodes in two patients), a HE re-staining was conducted. Under routine histology both lymph nodes were judged as negative.

Aspirates of 4 to 8 mL of bone marrow from the iliac crest were obtained from those patients who gave additional written consent for sampling bone marrow (59 patients) and were processed as previously described^[16]. The specimens were collected in heparin, and mononuclear cells isolated by density-gradient centrifugation through Ficoll-Hypaque (Pharmacia, Freiburg, Germany) at 400 r/min for 30 min, were deposited onto glass slides by cytocentrifugation at 150 r/min for 3 min. To detect tumor cells in bone marrow (Figure 2), we used the monoclonal antibody A45-B/B3 (IgG1; Micromet, Munich, Germany) that detects an epitope on a variety of cytokeratin components, including cytokeratins^[14,15,18,20].

Statistical analysis

All statistical calculations concerning survival (overall and recurrence-free survival) were based on the group of 89 patients who were available for follow-up. The primary outcome measure was the five-year survival probability. Secondary outcomes were the incidence of local recurrence and distant metastases of the disease. Survival was calculated from the date of resection until the date of death from any cause. For patients lost to follow-up, data were censored on the date the patient was last seen alive. Associations between categorical variables were assessed using Fisher's exact test. Survival estimates were derived using the method proposed by Kaplan and Meier^[35] and the log-rank test was used to assess differences in survival

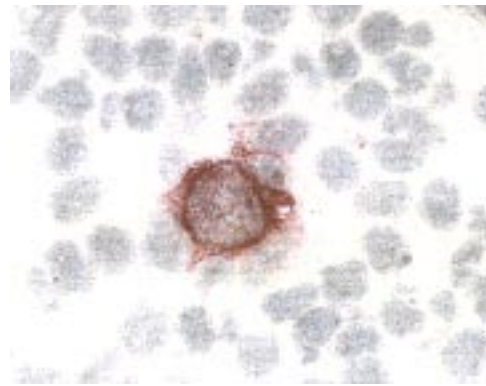


Figure 2 Immunohistochemically detectable bone marrow microinvolvement with monoclonal antibody AE1/AE3 ($\times 400$).

estimates among the groups. Point and interval estimates of the survival probabilities at 60 mo were calculated. For comparison purposes, log-rank test and exact stratified log-rank test were performed. Cox proportional-hazards modeling^[36] was used to investigate and adjust the major prognostic and stratification factors. $P < 0.05$ was considered statistically significant.

Since this analysis was intended to be explorative, no adjustment for multiple testing was carried out.

RESULTS

Characteristics of patients and comparison of staining procedures

One hundred and six patients [47 (44%) women and 59 (56%) men] with pancreatic ductal adenocarcinoma were included in the study. Their mean age was 61 years (range 32 to 83 years, median 61 years). Table 1 shows the characteristics of patients and tumors.

A total of 318 lymph nodes classified to be “tumor-free” by conventional histopathology were analyzed. Positive cells in the sinuses, the lymphoid interstitium, or in both locations were found in 132 lymph nodes (42%). These 132 positive lymph nodes were found from 73 (69%) of the 106 patients by immunostaining. Whereas the presence of Ber-EP4 cells was significantly associated with nodal metastases (pN1) identified through conventional histopathology ($P = 0.012$), no correlation between tumor stage and tumor grade was found.

Survival

After an average observation period of 18 mo (range 3 to 137 mo, median 12 mo), the presence of nodal microinvolvement was associated with significantly reduced recurrence-free and overall survival probabilities. The Kaplan-Meier overall survival curve for all patients who were stratified according to the presence or absence of occult tumor cells in lymph nodes showed a significant survival benefit for patients negative in immunohistochemistry (median not yet reached-NYR *vs* 13 mo; 2- year survival 66% *vs* 20%; 5-year survival 50% *vs* 0%) irrespective of the histopathological classification (pN0/pN1) of lymph nodes (log-rank test; $P < 0.0001$, Figure 3).

Table 1 Characteristics of patients and tumors

Variable	n	Ber-EP4 positive cells in lymph nodes (n)
All patients	106	73
Male	59	42
Female	47	31
Primary Tumor		
Carcinoma in situ	1	0
pT1	6	3
pT2	36	26
pT3	59	40
pT4	4	4
Nodal status		
Negative (pN0)	51	29*
Positive (pN1)	55	44*
Tumor Grade		
Well differentiated (G1)	5	4
Moderately differentiated (G2)	54	35
Poorly differentiated (G3)	47	34
Resection margin		
Negative (R0)	90	59
Positive (R1)	16	14
Tumor cells in Bone marrow		
Yes	14	9
No	45	31
Not analysed	47	33

Nodal status was detected both histopathologically and immunohistochemically. Bone marrow micrometastases were detected by immunohistochemistry. $P = 0.035$.

The analysis of the subset of patients who were staged pN0 in conventional histopathology revealed significantly better survival rates in patients without occult tumor cells as compared with those with nodal microinvolvement (median NYR *vs* 17 mo; 2-year survival 70% *vs* 34%; 5-year survival 61% *vs* 0%; log-rank test; $P=0.012$, Figure 4). Patients without any nodal involvement, as excluded by both conventional histopathology and immunohistochemistry, had a five-year overall survival probability of 61% (standard error: 13%). In contrast, the five-year survival probability of pN0-patients with nodal microinvolvement resembled that of pN1-patients (log-rank test; $P = 0.059$, Figure 4) and in both groups no patient was still alive 5 years after surgery.

The predictive value of nodal microinvolvement was strengthened by the finding that pN1-patients who additionally had disseminated tumor cells in other lymph nodes classified as tumor-free by histopathology had shorter recurrence-free and overall survival probabilities than pN1-patients without occult tumor cells in immunohistochemistry (median survival 33 *vs* 10 mo; 2-year survival 69% *vs* 10%; 5-year survival 69% *vs* 0%; log rank test: $P = 0.004$ and $P = 0.049$, respectively, data not shown).

Although no statistical significance was reached, pN1-patients *without* nodal microinvolvement had better overall survival probabilities (median survival = 33 mo) than pN0-patients *with* nodal microinvolvement (median survival = 17 mo). This could in part confirm the hypothesis that

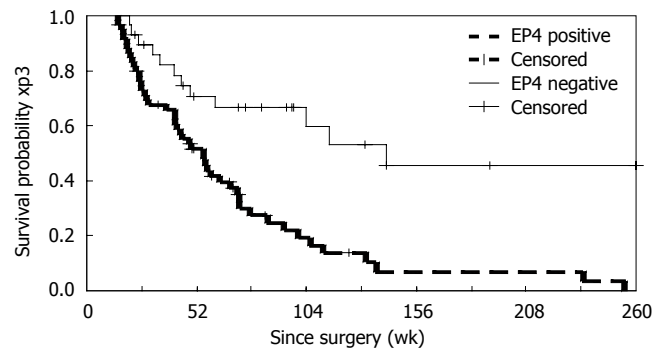


Figure 3 Overall survival according to the presence or absence of nodal microinvolvement in immunohistochemistry for patients alive at least 13 wk after surgery. Median: Not yet reached-NYR *vs* 13 mo; Mean: 81 (SD 15, 95% CI 52-109) *vs* 16 (SD 2, 95% CI 12-20); 2-yr overall survival 66% *vs* 20%; 5-yr overall survival 50% *vs* 0%.

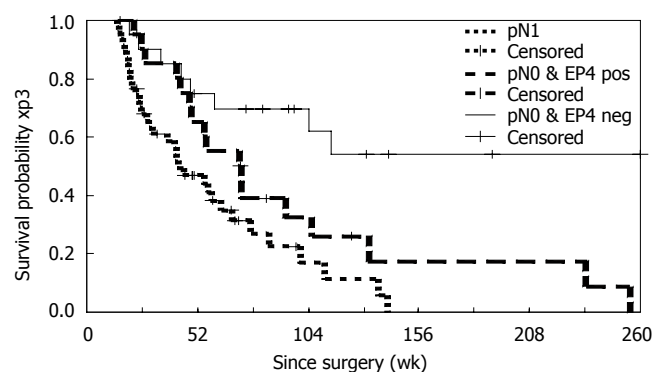


Figure 4 Overall survival according to the presence or absence of nodal metastases in conventional histopathology and immunohistochemistry for patients alive at least 13 wk after surgery. Median: Not yet reached-NYR *vs* 13 mo *vs* 10 mo; Mean: 90 (SD 15, 95% CI 60-119) *vs* 17 (SD 3, 95% CI 11-23) *vs* 14 (SD 2, 95% CI 7-13); 2-yr overall survival 66% *vs* 20%; 5-yr overall survival 50% *vs* 0%.

nodal microinvolvement in patients not burdened with nodal metastases detected through routine histology (pN0) might literally reflect systemic disease, whereas pN1-patients without nodal microinvolvement could be in fact treated as locally advanced disease. This should be discussed very cautiously since only 8 patients were included in the group of pN1-patients without nodal microinvolvement, but all of them were alive at the last follow-up.

Influence of resection margins

A total of 16 patients among the entire study population had positive resection margins (R1-status), only 2 of them belonged to the subset of pN0-patients without nodal microinvolvement. The remaining 14 patients with positive resection margins were either staged pN1 or had nodal microinvolvement which was as reported above, per se associated with a significantly worse prognosis. Due to this distribution of patients with R1-status, log-rank test might not have the sufficient power to assess the influence of the resection margins on overall survival. Therefore, no significant differences were found between patients with positive resection margins, as compared with those with negative resection margins ($P = 0.976$, data not shown).

Table 2 Cox regression analysis showing the presence of occult tumor cells in lymph nodes, tumor grade, and histopathologically detectable lymph node involvement as independent prognostic factors for disease-free and overall survival

Prognostic factor	Reference group	Relative risk	P	Lower bound	Upper bound
				95% confidence interval	
Recurrence-free (n = 86)					
Age at surgery		1.01	0.706	0.98	1.03
Sex	Male	1.42	0.223	0.81	2.49
Stage	pT1-pT2	0.87	0.652	0.48	1.57
Grade	G I - GII	3.14	0.000	1.74	5.68
Nodal involvement	pN0	1.66	0.112	0.89	3.10
Nodal microinvolvement (Ber-EP4)	Negative	2.92	0.005	1.39	6.13
Overall survival (n = 89)					
Age at surgery		1.00	0.996	0.97	1.03
Sex	Male	1.47	0.172	0.85	2.55
Stage	pT1-pT2	0.93	0.801	0.53	1.64
Grade	G I - GII	3.36	0.000	1.90	5.94
Nodal involvement	pN0	2.18	0.012	1.19	4.00
Nodal microinvolvement (Ber-EP4)	Negative	2.49	0.009	1.25	4.96

Influence of bone marrow micrometastases

Occult tumor cells were detected in 14 of 59 patients who were evaluated with respect to bone marrow micrometastases. No association was found between bone marrow and nodal microinvolvement (Fisher's exact test, $P = 0.75$). Among the 14 patients with bone marrow micrometastases, 9 (64%) had also nodal microinvolvement, whereas 5 (36%) did not. In turn, Ber-EP4-positive cells in lymph nodes were detected in 31 (69%) of the 45 patients with negative bone marrow findings (Table 1). Neither bone marrow micrometastases nor nodal microinvolvement was found in 14 patients. Here, we want to stress the finding (although again not significant due to the small number of patients) that those patients without any nodal involvement (negative both in histopathology and IHC) who had bone marrow micrometastases seemed to have considerably worse overall prognosis (mean survival time: 8 mo) than patients without (mean survival time: 75 mo). This also has to be discussed with considerable caution since we identified only 3 patients with bone marrow micrometastases (without any nodal involvement), although they all died within the first year unlike the other 8 patients without bone marrow micrometastases who were all alive on the last follow-up.

As regards the influence of bone marrow micrometastases alone, no significant differences were found with respect to relapse-free time ($P = 0.55$) and overall survival ($P = 0.14$), respectively. However, these results might be biased by the small number of patients analyzed.

Multivariate analysis

Apart from nodal involvement assessed either by histopathology or immunohistochemistry, the comparison of survival curves revealed also significant differences with respect to grading when G_{1,2} tumors were compared to G₃ tumors (median survival time: 28 mo *vs* 12 mo; log-rank test: $P < 0.001$).

Cox regression analysis identified the presence of occult tumor cells in lymph nodes, tumor grade, and histopathologically detectable lymph node involvement as

independent prognostic factors for disease-free and overall survival (Table 2). With respect to 5-year recurrence-free time, nodal microinvolvement had a relative risk of 2.92 (95% confidence interval: 1.39 to 6.13; $P = 0.005$), as compared with negative findings in immunohistochemistry. G₃ tumors had a relative risk of 3.14 as compared with G_{1/2} tumors (95% confidence interval: 1.74 to 5.68; $P = 0.000$) with respect to recurrence-free time. A pN1-stage, as compared to pN0 patients, carried a relative risk of 2.18 (95% confidence interval: 1.19 to 4.0; $P = 0.012$) as to overall survival time, but had no significant influence on recurrence-free time (relative risk of 1.66; 95% confidence interval: 0.89 to 3.10; $P = 0.112$). Age, sex and tumor stage had no independent prognostic influence on recurrence-free or overall survival.

The analysis of the interaction between pN-status, nodal microinvolvement and grading did not reveal that the proportional assumption was violated. Hence, the Cox model appeared appropriate and grading followed by nodal microinvolvement remained the two most important prognostic variables also in the subset of pN0-patients.

DISCUSSION

The key finding of this investigation is that isolated tumor cells, detectable in lymph nodes by immunohistochemical analysis, are strong independent prognosticators in pancreatic ductal adenocarcinoma irrespective of the histopathological N-status. We analyzed patients who suffered from pancreatic ductal adenocarcinoma and did not receive any adjuvant chemoradiation or chemotherapy. Two subsets of patients could be identified and were classified as pN0 in conventional histopathology. One subset with a poor five-year survival probability of 0% which was close to that of patients with overt nodal involvement (pN₁), the other subset had a much better prognosis with a five-year survival probability of over 50% without nodal microinvolvement, suggesting that immunohistochemistry can confirm the cardinal importance of occult tumor cells for the separation of the

respective survival curves in pN0-patients.

Even in patients who were staged as pN1, the detection of occult tumor cells in “tumor-free” lymph nodes had prognostic significance. This finding is consistent with previous observations of our group showing that survival is significantly worsened in both esophageal^[25] and non-small cell lung carcinoma^[32] when histopathological pN1-status is accompanied with nodal microinvolvement. Basically, pN1-status in solid tumors is considered as a local disease which can be potentially cured with surgery, although it generally carries a higher risk of systemic dissemination than pN0-status. Therefore, the finding that pN1 patients with additional nodal microinvolvement in “tumor-free” lymph nodes apart from overt lymph node metastases had significantly shorter recurrence-free survival as compared with pN1-patients without occult tumor cells suggests that immunohistochemistry may help to identify different risk profiles in these patients.

The reliability of these immunohistochemical assays used so far for detection of nodal microinvolvement is questioned^[16-26] and could be also hampered by a sampling error^[25,37]. The sampling error might be influenced by the number of lymph nodes dissected during the course of pancreatoduodenectomy and assessed by immunohistochemistry, as well as the number of lymph node sections and the level of these sections within the lymph nodes. In our present study, the lymph node dissection was performed as previously described by Pedrazzoli *et al*^[33]. A total of 1643 lymph nodes were removed with a median number of 16 (range 7 to 38) lymph nodes per patient. Thus, the first possible cause of a sampling error was diminished, considering the high median lymph node yield of 16 per patient. In another study (oesophagus carcinoma, not yet published) all lymph nodes dissected in the course of esophagectomy were immunohistochemically stained, unlike in this and some other previously published studies^[25] where only 20% of all lymph nodes were stained, showing comparable results in terms of impact on overall and relapse-free survival. We also believe that 6 sections cut from 3 different levels of each lymph node are enough for proper access to the nodal microinvolvement. Analyzing more than 3 sections would not be routinely feasible, and the positive correlation between the result of our assay and clinical outcome indicates that examining 3 lymph node levels are sufficient. The results from this study are in the same line with those published earlier from our group^[25], thus confirming that the random selection of lymph nodes for IHC is enough for access to the nodal microinvolvement.

The strong adverse influence of nodal microinvolvement on outcome was most likely the reason for the lack of prognostic significance of resection margins in this series. In the ESPAC-1 trial, the resection margin status ceased to be an independent, prognostic factor for overall survival when tumor grading and pN-status were co-variables in the regression modeling^[38], suggesting that the unfavorable outcome linked with poorly differentiated tumors with nodal metastases can hardly be impaired by further adverse variables, e.g. R1-status. We therefore assume that the characteristics of patients with R1-status are considerably biased. This hypothesis is strengthened by

our observation that out of the 16 patients with R1-status all except two had occult tumor cells in immunohistochemistry.

Our data also indicate primary dissemination of tumor cells into lymph nodes before blood-borne spread occurs, because only 22% of the patients with nodal microinvolvement had identifiable tumor cells in their bone marrow. The lack of a significant difference in recurrence-free time between patients with nodal microinvolvement alone and those with additional involvement of the bone marrow suggests that the key event in pancreatic cancer progression is the spread of tumor cells to the regional lymph nodes. Nodal microinvolvement seems to indicate a systemic disease in pancreatic carcinoma much more accurately than occult tumor cells in bone marrow. However, these results have to be interpreted cautiously because bone marrow findings may have been biased by the fact that only a subset of patients was analyzed. Therefore, the influence of bone marrow microinvolvement on the outcome of patients with pancreatic carcinoma needs to be clarified in future studies.

Although chemoradiation and/or chemotherapy for adjuvant treatment of pancreatic carcinoma may have severe side effects^[39], in common clinical practice, it is in most instances applied irrespective of tumor stage. This reflects the distrust in the value of conventional tumor-staging nomenclature in terms of reliably predicting the risk of tumor relapse even in patients with early pancreatic cancer (T₁, N₀). Our data indicate that immunohistochemical assessment of lymph nodes can be used to refine the staging system for pancreatic ductal adenocarcinoma and might help us to identify patients who could not be cured by surgery alone and need adjuvant therapy. In turn, patients who are true node-negative both in histopathology and in immunohistochemistry have an excellent five-year survival probability of nearly 60%, even without chemotherapy. Whether this prognosis can be further improved by adjuvant therapy needs further study.

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RAPID COMMUNICATION

Phase II study of protracted irinotecan infusion and a low-dose cisplatin for metastatic gastric cancer

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CONCLUSION: This regimen showed a high level of activity and acceptable toxicity in patients with metastatic gastric cancer.

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Key words: Gastric cancer; CPT-11; CDDP; Protracted Irinotecan; Chemotherapy

Imamura H, Ikeda M, Furukawa H, Tsujinaka T, Fujitani K, Kobayashi K, Narahara H, Kato M, Imamoto H, Takabayashi A, Tsukuma H. Phase II study of protracted irinotecan infusion and a low-dose cisplatin for metastatic gastric cancer. *World J Gastroenterol* 2006; 12(40): 6522-6526

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Abstract

AIM: To test protracted irinotecan infusion plus a low-dose cisplatin in this Phase II trial to decrease its toxicity.

METHODS: The eligibility criteria were: (1) histologically proven measurable gastric cancer; (2) performance status of 0 or 1; (3) no prior chemotherapy or completion of prior therapy at least 4 wk before enrollment; (4) adequate function of major organs; (5) no other active malignancy; and (6) written informed consent. The regimen consisted of irinotecan (60 mg/m²) on d 1 and 15 by 24-h infusion and cisplatin (10 mg/m²) on d 1, 2, 3, 15, 16, and 17. Treatment was repeated every 4 wk.

RESULTS: Thirty-one patients were registered between April 2000 and January 2001. The response rate for all 31 patients, 20 patients without prior chemotherapy, and 11 patients with prior chemotherapy was 52% (16/31), 60% (12/20), and 36% (4/11), respectively. The median survival time was 378 d. The median number of courses given to all patients was 2. Grade 4 neutropenia occurred in 11 (35%) patients, while grade 3 to 4 diarrhea or nausea occurred in 1 (3%) and 3 (10%) patients, respectively. Fatigue was minimal as grade 1 fatigue was found only in 3 (10%) patients. Other adverse events were mild and no treatment-related deaths occurred.

INTRODUCTION

In Japan, for advanced gastric cancer, surgery is still the most effective treatment and good survival can be achieved if the tumor is resectable. On the contrary, unresectable advanced or recurrent gastric cancer still has a poor prognosis and chemotherapy is the most important treatment for survival prolongation. To date, combination chemotherapy with 5-fluorouracil (5-FU) and cisplatin has been used most widely. This two-drug regimen showed superior response rate in comparison with single-agent 5FU regimen, however, failed to demonstrate survival prolongation^[1-3]. This regimen has response rates ranging from 10% to 35%, and the median survival time (MST) from 6 to 8 mo with around 10% in a 2-year survival^[4]. Advent of new active agents is awaited.

Among new drugs, irinotecan, a derivative of camptothecin, has strong antitumor activity through inhibition of DNA topoisomerase I^[5]. It has a single agent activity with a response rate of 23.3%^[6]. As the response rate is not satisfactory, irinotecan was first investigated in combination with cisplatin. Phase I study was conducted for metastatic gastric cancer and treatment regimen of irinotecan (70 mg/m², d 1 and 15) and cisplatin (80 mg/m², d 1), every 4 wk, was recommended^[7]. Boku's group tested this regimen for metastatic gastric cancer^[8]. The overall response rate was 48% and the median survival time was 272 d. Although no treatment-related deaths occurred, adverse events were severe, including grade 4 neutropenia in

57% of the patients and grade 3 or 4 diarrhea in 20%. As for renal toxicity, they found a total of 34% (grade 1: 23%, grade 2: 11%) serum creatinine increase. This combination study was followed with modification of weekly schedule to reduce toxicity in western countries. Ajani *et al*^[9] conducted phase II study with irinotecan (65 mg/m²) with cisplatin (30 mg/m²), both administered intravenously 1 d per week for 4 consecutive weeks, followed by two weeks recovery period. Of the 36 patients registered, 21 achieved complete or partial response with response rate of 58%. They had one treatment-related death of neutropenic sepsis with multiple organ failure. Major toxic effects were diarrhea, neutropenia, and fatigue. The incidence of grade 3 or 4 neutropenia was 37%, however, grade 3 or 4 diarrhea was still found in 22% of patients. Surprisingly, grade 3 or 4 fatigue was 41%, a cause of delays or cancellation of drugs. To decrease adverse events, Fujitani *et al*^[10] conducted a pharmacokinetic study of continuous infusion of irinotecan for 24 h combined with infusion of cisplatin over 90 min, and demonstrated only mild hematological and nonhematological adverse reactions, while protracted infusion of irinotecan increased the area under the concentration *vs* time curve (AUC) of SN-38.

Accordingly, we further investigated the safety and efficacy of this combined protracted infusion of irinotecan (to maintain a high AUC of SN-38) with a low-dose cisplatin to determine whether this regimen can improve response rate and reduce toxicity.

MATERIALS AND METHODS

Eligibility criteria

Patients were required to satisfy the following eligibility criteria; (1) histologically proven gastric cancer; (2) measurable metastatic lesions; (3) Eastern Clinical Oncology Group scale performance status of 0 or 1; (4) no prior chemotherapy or completion of therapy at least 4 wk before entry; (5) adequate function of the bone marrow WBC count $\geq 4 \times 10^9$ and $\leq 12 \times 10^9$, platelet count $\geq 100 \times 10^9$, and hemoglobin ≥ 95 g/L, liver (serum bilirubin ≤ 25.6 μ mol/L and serum transaminases ≤ 1667 nkat/L), and kidneys (serum creatinine ≤ 133 μ mol/L); (6) normal cardiac function; (7) no other severe medical conditions; (8) no other active malignancy; and (9) ability to give written informed consent. This study was approved by the institutional review boards of all participating hospitals.

Treatment schedule

On d 1, irinotecan (60 mg/m²) was administered as a 24-h infusion; the drug was diluted in 500 mL of saline or 50 g/L glucose and was protected from the light. Cisplatin (10 mg/m²) was administered as a 60-min intravenous infusion with adequate hydration on d 1, 2, and 3. The same doses of irinotecan and cisplatin were repeated on d 15 and 15-17, respectively, to complete one course. Treatment was repeated every 4 wk until the occurrence of disease progression, patient refusal, or unacceptable adverse reactions. On d 15, if the patient had leucopenia or thrombocytopenia of grade 2 or higher, diarrhea of grade 1 or higher, or fever (a temperature $> 38^\circ\text{C}$) due to infection, administra-

tion of the second dose of irinotecan was delayed for one week. If recovery from the adverse reaction did not occur after one week, the second dose was skipped. If a grade 4 hematologic adverse event, grade 3 or 4 diarrhea, fever associated with infection, or omission of the second dose occurred, the dose of irinotecan for the second course was reduced to 50 mg/m². The antiemetic granisetron was given before cisplatin administration. Granulocyte colony-stimulating factor (G-CSF) was used when grade 4 leucopenia and/or neutropenia occurred. If the patient stopped treatment due to toxicity or tumor progression, other chemotherapy or surgery was offered.

Evaluation

The National Cancer Institute Common Toxicity Criteria (Version 2.0) were applied for the assessment of adverse events. The objective response of measurable lesions was evaluated by standard World Health Organization criteria. Both patient eligibility and the response to treatment were reviewed extramurally.

Statistical analysis

The expected efficacy rate of this regimen was hypothesized to be 50%, so the required number of patients was 25 when the 95% confidence interval was set at $\pm 20\%$. Because some patients might be excluded from analysis, the target number of patients for this study was set at 30. Analysis was performed on an intent-to-treat basis. The percentage of patients with complete remission (CR) plus partial remission (PR) among all treated patients was defined as the response rate. The 95% confidence interval (CI) of the response rate was calculated using the normal approximation and precision method. The Kaplan-Meier method was used to calculate the survival period.

RESULTS

Patients characteristics

Thirty-one patients were enrolled from April 2000 to January 2001. One patient was found to be ineligible because the performance status was 2. As analysis was performed on an intent-to-treat basis, this patient was also analyzed for efficacy and safety, so all 31 patients were assessed for efficacy and safety. Their clinical characteristics are shown in Table 1. The median age was 60.5 (range: 26-75 years) and 21 patients (68%) had performance status of 0. Sixteen patients (52%) had intestinal type adenocarcinoma and 14 patients had diffuse tumors. The measurable metastatic lesions were located in the lymph nodes in 20 patients (26 lesions), liver in 13 patients, peritoneum in 3 patients, ovary in 2 patients, and the skin, lung, and rectum in one patient each, respectively. Twenty patients (65%) had not received prior chemotherapy, while the other 11 patients had undergone chemotherapy with 5-FU, cisplatin plus 5-FU, or other drugs. All of the patients who had received prior therapy showed progressive disease before enrollment. The total number of treatment courses was 84 with a median number of 2 cycles per patient (range 1-5). A total of 149 of 168 planned CPT-11 administration was carried out. While a total of 112 of 122 (92%) planned CPT-11 was administered in the first and second cycle, a

Table 1 Patient profile

	<i>n</i>	Site of metastases	
Sex			
Female	20	Lymph nodes	26
Male	11	Perigastric	6
PS		Para-aortic	6
0	21	Virchow's	3
1	9	Neck	2
2	1	Mediastinum	1
Histology		Others	8
Intestinal	16	Liver	13
Diffuse	14	Peritoneal	3
Others	1	Skin	1
Prior chemotherapy		Lung	1
No	20	Ovary	2
Yes	11	Rectum	1

PS: Performance status.

Table 2 Response to therapy *n* (%)

	CR	PR	NC	PD	RR	95% CI
Overall	0	16 (52)	11 (35)	4 (13)	16 (52)	33-70
Prior chemotherapy						
Yes	0	4 (36)	4 (27)	3 (27)	4 (36)	11-69
No	0	12 (60)	7 (35)	1 (5)	12 (60)	36-81
Liver	2 (15)	5 (39)	5 (39)	1 (8)	7 (54)	
Lymph Nodes	2 (8)	12 (46)	12 (46)	0	14 (54)	
Peritoneal	0	1 (33)	1 (33)	1 (33)	1 (33)	
Ovary	0	0	1 (50)	1 (50)	0	
Lung	0	1 (100)	0	0	1 (100)	
Others	0	1 (50)	1 (50)	0	1 (50)	

CR: Complete remission; PR: Partial response; NC: No change; PD: Progressive disease; RR: Response rate.

total of 37 of 46 (80%) planned CPT-11 administration in the third to fifth cycles. Dose reduction was not required in the first cycle, but it was needed in 8 patients in the second cycle and one patient in the third cycle. Causes of dose reduction were grade 4 neutropenia in 5 patients, grade 4 leukopenia in one patient, and second dose skip in 3 patients. Treatment was stopped due to tumor progression in 8 patients, refusal to continue in 9 patients, and insufficient recovery from adverse reactions in 3 patients. Median duration between the beginning and end of treatment was 75 d. The actual administered dose of CPT-11 in the all courses was 25.7 mg/m² per week and that of cisplatin was 13.0 mg/m² per week, which corresponded to 85.5% and 86.4% of the planned doses.

Response and survival

There were no complete remissions, but partial remission was achieved in 16 patients for a response rate of 52% (16/31 patients, 95% CI: 33% to 70%). The response rate was 60.0% for the 20 patients without prior chemotherapy (12/20 patients, 95% CI: 36% to 81%), while the rate for the 11 patients with prior chemotherapy was only 36% (4/11 patients, 95% CI: 11% to 69%). The response rate of patients with lymph node, liver, and peritoneal

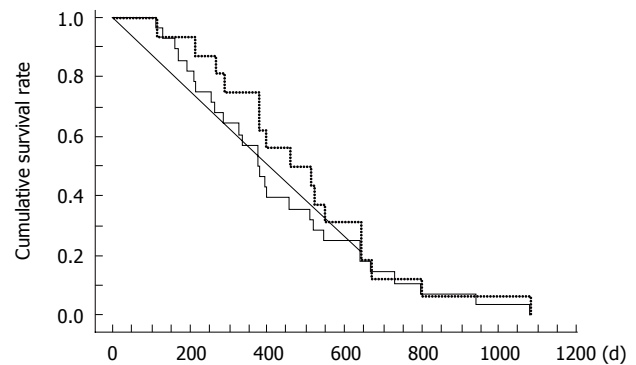


Figure 1 Survival curves of all patients and patients without prior chemotherapy. —: Survival curve of all patients (median survival time, 378 d).: Survival curve of patients without prior chemotherapy (median survival time, 509 d).

Table 3 Adverse reactions

	Grade (%)			
	1	2	3	4
Hematologic				
Leukopenia	1 (3)	14 (45)	8 (26)	3 (10)
Neutropenia	1 (3)	2 (6)	13 (42)	11 (35)
Thrombocytopenia	8 (25)	1 (3)	1 (3)	1 (3)
Anemia	5 (16)	13 (42)	7 (23)	2 (6)
Nonhematologic				
Fatigue	3 (10)	0	0	0
Nausea	12 (39)	8 (26)	3 (10)	0
Diarrhea	9 (29)	4 (13)	1 (3)	0
Abdo pain	2 (6)	2 (6)	1 (3)	0
Alopecia	11 (35)	10 (30)	1 (3)	0
AST	1 (3)	0	0	0
T. bilirubin	1 (3)	0	0	0
Cr	8 (26)	0	0	0

Abdo pain: Abdominal pain.

metastasis were 54% (14/26 patients), 54% (7/13 patients) and 33% (1/3 patients), respectively (Table 2). The median duration of response for all the patients was 218 d. The median survival time of all patients was 378 d, while the median survival time of the 20 patients without prior chemotherapy was 509 d. The 1-year and 2-year survival rates were 57% (95% CI: 37%-72%) and 9% (95% CI: 2%-23%), respectively (Figure 1).

Adverse reactions

Adverse reactions to this regimen are shown in Table 3. Grade 4 neutropenia, leukopenia, anemia, and thrombocytopenia were observed in 11 (35%), 3 (10%), 2 (6%), and 1 (3%) of the patients, respectively. The median nadir of the neutrophil count was seen on d 8 (range: d 2-19). G-CSF was administered to 15 patients (48%) during 28 courses. Non-hematologic adverse reactions (nausea, diarrhea, increased AST, and increased bilirubin) were all moderate, with grade 3 nausea (10%) and grade 3 diarrhea (3%) being the maximal reactions. Loperamide or other mild anti-diarrheal medicines were effective for diarrhea, while granisetron and other common anti-emetic medicines were effective for nausea without additional hydration. Renal

toxicity determined by the level of serum creatinine was also mild. We found 8 patients (26%) with only grade 1 abnormal creatinine level. Fatigue was found in only 3 patients and all grade 1. The non-hematologic reactions did not disrupt the treatment schedule and there were no treatment-related deaths.

DISCUSSION

The aim of this phase II study was to confirm the antitumor effects and safety of combined chemotherapy of protracted infusion of irinotecan and low-dose cisplatin^[10]. Protracted infusion of irinotecan significantly increases the AUC of active metabolite, SN-38^[10], suggesting it has the potential to maximize the effect of irinotecan. The response rate to irinotecan as a single agent for gastric cancer was reported to be 23.3%^[6] and that for cisplatin was 20%^[11]. The overall response rate of the 31 patients in the present series was 52% and that of 20 patients without any prior chemotherapy was 60%, with both rates being better than for single agent therapy. These data are consistent with a previous phase II study of cisplatin and irinotecan therapy^[8,9], and confirms the activity of combined therapy of irinotecan and cisplatin.

Diarrhea is a serious toxicity of irinotecan therapy. The incidence of grade 3 or 4 diarrhea was reported to be 18.8% or 35.6% with irinotecan alone and 21.9% or 35.6% with bolus combined administration^[12-15]. In the previous phase II studies of irinotecan and cisplatin, diarrhea was still one of the major cause that could affect treatment schedule and the incidence of grade 3 or 4 diarrhea was around 20%^[8,9]. In the present study, grade 4 diarrhea did not occur and only one patient (3%) suffered from grade 3 diarrhea that did not necessitate a change of schedule.

The incidence of grade 3 or 4 neutropenia (77%) is still high in this study, and Boku *et al*^[8] found the incidence of grade 3 or 4 neutropenia was 89%. Ajani *et al*^[9] demonstrated that 37% grade 3 or 4 neutropenia by weekly administration. Sakaki *et al*^[16] found a positive correlation between the AUC of irinotecan and the decrease of the white cell count, however, diarrhea has a stronger correlation with the AUC of SN-38 than with that of irinotecan. As this treatment regimen has greater AUC of SN-38 while AUC of irinotecan was same in comparison with 90-min infusion of irinotecan^[10], we expected to find more hematological toxicities than diarrhea. To the contrary, we found significantly less common severe diarrhea, and high incidence of grade 3 or 4 neutropenia that was comparable to the previous Japanese trial^[8]. The target dose of irinotecan for this study ($60 \text{ mg/m}^2 \times 2$) was lower than that used by Boku ($70 \text{ mg/m}^2 \times 2$)^[8] or that used to treat lung cancer ($60 \text{ mg/m}^2 \times 3$)^[17,18]. Actual dose of CPT-11 administered in the Boku's study was 28.5 mg/m^2 per week, and that in our study was 25.7 mg/m^2 per week, so our regimen seemed to reduce the incidence and grade of diarrhea while achieving a similar response rate with a lower dose of irinotecan.

Nausea and renal toxicity is a common problem with cisplatin therapy, so we administered a low dose of 10 mg/m^2 on six occasions to achieve a total dose of 60 mg/m^2 . Grade 3 nausea occurred in 3 patients (10%) and there

was no grade 4 nausea. Low-dose, repeated administration has already been reported to decrease the incidence and grade of nausea due to cisplatin^[19-22]. Some Japanese authors have reported that repeated cisplatin administration at a low dose reduces the incidence of nausea and allows outpatient treatment, and that this method achieves a high response rate and longer survival when combined with irinotecan without the need for 24-h infusion of the latter drug^[9,23-25]. As for renal toxicity, Boku *et al* with high-dose cisplatin administration^[8] found grade 1 or 2 serum creatinine increase in 23 and 11 % of patients, respectively. We found only grade 1 abnormality, and the incidence of 26%, demonstrating that renal toxicity with this regimen is minimal. As hydration is not required to prevent renal toxicity with low-dose therapy^[20], continuous infusions are not necessary after finishing irinotecan administration on d 2, 3, 16, and 17.

In order to complete this regimen, hospitalization is required to receive 24-h irinotecan infusions and 6 divided dose of cisplatin. Therefore, this regimen is not applicable for out-patient basis; however, as the incidence of fatigue of this regimen was 10% (only grade 1), severe diarrhea was rare, and hydration was not necessary, it is possible to treat patients on an out-patient basis between infusions. Ajani *et al* demonstrated very high incidence (41%) of grade 3 or 4 fatigue with weekly irinotecan and cisplatin administration, and they discussed cisplatin might contribute to excessive fatigue, thus either the dose of cisplatin might be reduced, or cisplatin might be replaced by other agents. Our results with 10% grade 1 fatigue suggested that low dose cisplatin is a practical alternative for reducing the fatigue.

In conclusion, though hospitalization is required at this time, 24-h infusion of irinotecan combined with a low-dose, repeated administration of cisplatin achieved a high response rate and prolonged the survival of patients with metastatic gastric cancer. This regimen also reduces non-hematologic adverse reactions and thus shortens the time in hospital.

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Gallbladder carcinoma associated with pancreatobiliary reflux

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Abstract

AIM: To detect the patients with and without pancreaticobiliary maljunction who had pancreatobiliary reflux with extremely high biliary amylase levels.

METHODS: Ninety-six patients, who had diffuse thickness (> 3 mm) of the gallbladder wall and were suspected of having a pancreaticobiliary maljunction on ultrasonography, were prospectively subjected to endoscopic retrograde cholangiopancreatography, and bile in the common bile duct was sampled. Among them, patients, who had extremely high biliary amylase levels ($> 10\,000$ IU/L), underwent cholecystectomy, and the clinicopathological findings of those patients with and without pancreaticobiliary maljunction were examined.

RESULTS: Seventeen patients had biliary amylase levels in the common bile duct above $10\,000$ IU/L, including 11 with pancreaticobiliary maljunction and 6 without pancreaticobiliary maljunction. The occurrence of gallbladder carcinoma was 45.5% (5/11) in patients with pancreaticobiliary maljunction, and 50% (3/6) in those without pancreaticobiliary maljunction.

CONCLUSION: Pancreatobiliary reflux with extremely high biliary amylase levels and associated gallbladder carcinoma could be identified in patients with and without pancreaticobiliary maljunction, and those patients might be detected by ultrasonography and bile sampling.

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Key words: Amylase; Bile; Gallbladder carcinoma; Pancreatobiliary reflux; Pancreaticobiliary maljunction; Diagnosis

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INTRODUCTION

It is well known that pancreatobiliary reflux is an important risk factor for the carcinogenesis of the biliary system in patients with pancreaticobiliary maljunction (PBM)^[1,2], which is a congenital anomaly defined as an abnormal union of the common bile duct and pancreatic duct that is located outside the duodenal wall where a sphincter system is not present and pancreatic juice freely regurgitates into the biliary tract through the communication^[3].

Recently, we reported that pancreatobiliary reflux with extremely high biliary amylase levels can occur not only in patients with PBM, but also in those without PBM^[4,5], although the latter condition is not well known yet. In the present study, we tried to detect the patients, who had pancreatobiliary reflux with extremely high biliary amylase levels, and examined the clinicopathological findings of those patients.

MATERIALS AND METHODS

Subjects

Between March 2002 and February 2006, 96 patients, who had diffuse thickness of the gallbladder wall above 3 mm and were suspected of having pancreaticobiliary maljunction on ultrasonography, were prospectively subjected to endoscopic retrograde cholangiopancreatography (ERCP). Bile in the common bile duct was sampled as follows and biliary amylase levels were measured in those patients. After selective insertion of an ERCP catheter into the bile duct, bile was aspirated from the common bile duct at a depth of 5 cm. To avoid contamination with pancreatic juice, the first 5 mL of the aspirated bile was not used to measure biliary amylase levels, and another sample of bile was obtained for the measurement. In all patients, serum amylase was measured within 48 h before cholecystectomy. Amylase in the serum and bile was measured by an enzymatic method using 3-ketobutylidene-2-chloro-4-nitrophenyl-maltopentaoside (Diacolor Neonate, Toyobo, Osaka, Japan) as the substrate^[6]. The normal range for serum amylase in our institution is 130 to 400 IU/L. We defined extremely high biliary amylase levels as those above $10\,000$ IU/L, and patients with extremely high biliary amylase levels were indicated for cholecystectomy. Patients having bile duct stenosis, filling defect in the bile duct, including choledocholithiasis on ERCP, were excluded

Table 1A Clinical characteristics of patients with non-pancreaticobiliary maljunction

	Age	Sex	Biliary amylase (IU/L)	Common channel length (mm)	Pathology	Depth of invasion	GB stone	Choledocal cyst
1	56	M	47190	7	Hyperplasia		+	-
2	69	F	27038	8	Hyperplasia		+	-
3	63	F	61710	4	Hyperplasia		-	-
4	53	F	52600	8	ca	m	-	-
5	68	F	84700	9	ca	m	-	-
6	70	F	64260	4	ca	ss	-	-

GB: Gallbladder; m: Tumor invades the mucosa; ss: Tumor invades the subserosa; ca: Adenocarcinoma.

Table 1B Clinical characteristics of patients with pancreaticobiliary maljunction

	Age	Sex	Biliary amylase (IU/L)	Common channel length (mm)	Pathology	Depth of invasion	GB stone	Choledocal cyst
1	65	F	12030	18	chr-itis		+	+
2	74	F	153000	16	chr-itis		+	-
3	63	M	136400	48	ADM		-	+
4	34	F	145000	15	Hyperplasia		+	-
5	46	F	103500	26	Hyperplasia		-	-
6	40	F	126900	28	Hyperplasia		+	+
7	47	M	95400	19	ca	m	-	-
8	54	F	132180	27	ca	ss	-	-
9	64	F	177520	24	ca	ss	-	-
10	75	F	26510	20	ca	ss	-	-
11	57	F	21100	19	ca	se	-	-

GB: Gallbladder; m: Tumor invades the mucosa; ss: Tumor invades the subserosa; se: Tumor perforates serosa; chr-it is: Chronic cholecystitis; ADM: Adenomyomatosis; ca: Adenocarcinoma.

from the present study, because their biliary amylase levels in the common bile duct would not correctly reflect pancreatobiliary reflux.

Patients were divided into those with PBM and without PBM (non-PBM). On ERCP images, the two ducts were always communicated in PBM patients, but not in non-PBM patients due to the sphincter contraction. The length of the common channel was measured on ERCP images corrected for magnification using the diameter of the endoscope as a reference.

Informed consent was obtained from all the patients. The study protocol was approved by the ethics committee of our institution.

Tissue specimens and histologic analysis

All specimens were fixed in 10% buffered formalin. The gallbladder tissues embedded in paraffin were cut into 4-μm serial sections, and the gallbladder mucosa was histologically examined using sections stained with hematoxylin and eosin (HE). All specimens were diagnosed based on light microscopic findings. Carcinoma of the gallbladder was diagnosed according to the criteria reported by Albores-Saavedra *et al*^[7].

RESULTS

Serum amylase was within 600 IU/L in all patients.

Among the 96 patients who underwent ERCP, bile was successfully sampled in 87 patients; four patients underwent unsuccessful ERCP, two had bile duct stenosis, three had choledocholithiasis, and these patients were excluded from the present study. Consequently, 17 patients had extremely high biliary amylase levels in the common bile duct above 10000 IU/L, including 11 PBM patients and 6 non-PBM patients. Among the PBM patients, 4 had a choledocal cyst. All patients with biliary amylase levels above 10000 IU/L underwent cholecystectomy. The main clinical characteristics of PBM and non-PBM patients are shown in Tables 1 and 2. The occurrence of gallbladder carcinoma was 45.5% (5/11) in PBM patients, and 50% (3/6) in non-PBM patients. PBM patients included 1 carcinoma limited within the mucosa, while non-PBM patients included 2 (Table 1, Figure 1).

DISCUSSION

Gallbladder carcinoma is known to carry a poor prognosis^[8]. It is therefore essential to identify patients at a high risk for developing gallbladder carcinoma^[9]. The risk of gallbladder carcinoma associated with PBM is substantial; it was reported that the occurrence of biliary cancer in 388 PBM patients without biliary dilatation was 37.9%, including 93.2% with gallbladder carcinoma and 6.8% with bile duct cancer, while that in 1239 PBM

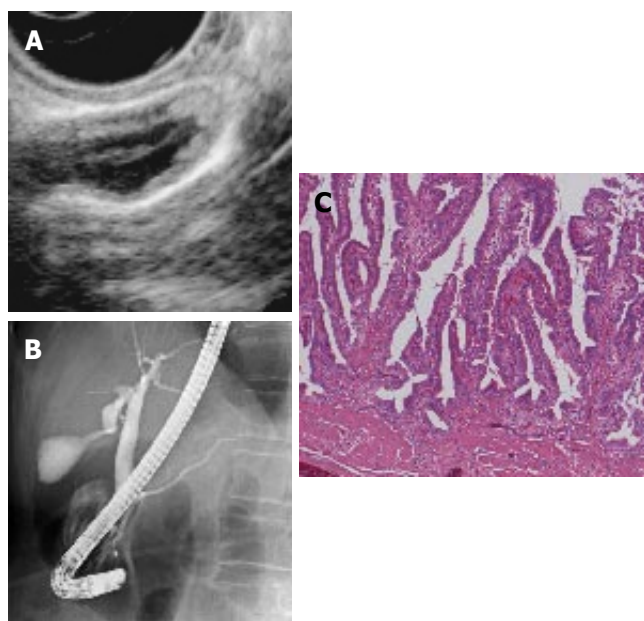


Figure 1 The 52-year old woman who had extremely high biliary amylase level without pancreaticobiliary maljunction and gallbladder carcinoma limited within the mucosa. **A:** Ultrasonography, showing diffuse thickness of the gallbladder wall above 3 mm; **B:** ERCP shows no pancreaticobiliary maljunction and common channel of 7 mm. Biliary amylase levels in the common bile duct was 52 600 IU/L; **C:** Gallbladder carcinoma limited within the mucosa (HE, x 40).

patients with choledocal cyst was 10.6%, including 33.6% with extrahepatic bile duct cancer and 64.9% with gallbladder carcinoma^[10]. Numerous studies have shown that pancreatobiliary reflux is a major risk factor for biliary carcinogenesis in patients with PBM; the mixture of bile and pancreatic juice can induce chronic inflammation and genetic alterations and increase cellular proliferation of the biliary tract epithelium, leading to hyperplasia, dysplasia and ultimately carcinoma of the biliary tract mucosa^[11,12].

Pancreatobiliary reflux with extremely high biliary amylase levels was also identified in non-PBM patients in the present study, and 50% of those patients had gallbladder carcinoma. Furthermore it included gallbladder carcinoma limited within the mucosa, and thus detection of pancreatobiliary reflux with extremely high biliary amylase levels might allow management for gallbladder carcinoma at an early stage.

In order to identify pancreatobiliary reflux pre-operatively, secretin-injection magnetic resonance cholangiopancreatography (MRCP) is an option that has proved useful as we have previously reported^[4,5], although high amylase levels in the bile sampled during ERCP is a direct evidence of pancreatobiliary reflux. One of the characteristic findings of ultrasonography associated with pancreaticobiliary maljunction was reported as diffuse thickness of the gallbladder wall above 3 mm, that might reflect mucosal change associated with pancreatobiliary reflux including chronic inflammation and increased cellular proliferation of the gallbladder mucosa^[13]. In the present study, ultrasonographic finding also proved useful to diagnose non-PBM patients with extremely high biliary amylase levels, although the finding was non-specific and was seen in other diseases including chronic cholecystitis with or without gallstone, adenomyomatosis, and liver cirrhosis^[13].

Table 2 Clinicopathological findings in patients with and without pancreaticobiliary maljunction

	non-PBM (n = 6)	PBM (n = 11)
Age (mean ± SD)	63.2 ± 7.2	54.1 ± 12
Female (number)	5/6 (83%)	9/11 (82%)
CBD-Amy (mean ± SD) IU/L	56 250 ± 19 246	102 686 ± 57 660
Cholecystolithiasis (number)	2/6 (33%)	4/11 (36%)
Length of the common channel (mean ± SD) mm	6.7 ± 2.2	23.6 ± 9.2
Gallbladder carcinoma (number)	3/6 (50%)	5/11 (46%)

PBM: Pancreatobiliary maljunction; CBD-Amy: Amylase levels in the common bile duct; SD: Standard deviation.

Reflux of pancreatic juice into the biliary tract is influenced by the function of Oddi's sphincter and the form of the junction of the pancreaticobiliary duct^[14]. One of the mechanisms of pancreatobiliary reflux in non-PBM patients could be a long common channel. Actually, 67% (4/6) of non-PBM patients had a long common channel more than 7 mm in the present study. Misra *et al* reported that a common channel more than 8 mm in length was seen more frequently in patients with gallbladder carcinoma (38%) compared with normal subjects (3%) or patients with gallstones (1%)^[15]. Kamisawa *et al* also reported that the occurrence of gallbladder carcinoma in non-PBM patients with a common channel of more than 6 mm in length was 12%, being significantly higher than that in controls^[16]. They speculated that the longer common channel could be associated with a higher occurrence and a more significant degree of pancreatobiliary reflux, which might be the cause of gallbladder carcinoma, although they did not measure the biliary amylase levels in their study. Another mechanism of pancreatobiliary reflux in non-PBM patients, especially in our two patients with a common channel of 4 mm, might be the dysfunction of the sphincter of Oddi, however, the precise mechanism of pancreatobiliary reflux in non-PBM patients should be further clarified in future studies.

In conclusion, pancreatobiliary reflux with extremely high biliary amylase levels and associated gallbladder carcinoma could be identified in patients with and without pancreaticobiliary maljunction, and those patients might be detected by ultrasonography and bile sampling.

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Signaling pathway of insulin-like growth factor- II as a target of molecular therapy for hepatoblastoma

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Abstract

AIM: To address the possibility that insulin-like growth factor (IGF)- II is a growth factor and its signaling pathway so as to develop a molecular therapy for hepatoblastoma.

METHODS: Huh-6 and HepG2, human hepatoblastoma cell lines, were used. IGF- II was added to the medium deprived of serum. Western blot analysis was performed to clarify the expression of IGF-I receptor (IGF-IR). Inhibitors of IGF-IR (picropodophyllin, PPP), phosphatidyl-inositol (PI) 3-kinase (LY294002 and Wortmannin), or mitogen-activated protein (MAP) kinase (PD98059) were added to unveil the signaling pathway of IGF- II. Cells were analyzed morphologically with hematoxylin-eosin staining to reveal the mechanism of suppression of cell proliferation.

RESULTS: IGF- II stimulated cells proliferated to 2.7 (269% \pm 76%) (mean \pm SD) (Huh-6) and 2.1 (211% \pm 85%) times (HepG2). IGF-IR was expressed in Huh-6 and HepG2. PPP suppressed the cell number to 44% \pm 11% (Huh-6) and 39% \pm 5% (HepG2). LY294002 and Wortmannin suppressed the cell number to 30% \pm 5% (Huh-6), 44% \pm 0.4% (HepG2), 49% \pm 1.0% (Huh-6) and 46% \pm 1.1% (HepG2), respectively. PD98059 suppressed the cell number to 33% \pm 11% for HepG2 but not for Huh-6. When cell proliferation was prohibited, many Huh-6 and HepG2 cells were dead with pyknotic or fragmented nuclei, suggesting apoptosis.

CONCLUSION: IGF- II was shown to be a growth factor of hepatoblastoma *via* IGF-I receptor and PI3 kinase which were good candidates for target of molecular therapy.

INTRODUCTION

Hepatoblastoma (HBL) arises in the liver of infants younger than 3 years of age^[1]. The growth factor and its signaling pathway could be revealed so as to develop a novel molecular therapy for HBL.

Insulin-like growth factor (IGF)- II is a hormone that plays an important role in fetal growth and development. IGF- II is abundantly expressed in fetus, with its concentration decreasing after birth^[2]. Interestingly, IGF- II is detected at high levels in the serum of HBL patients^[3]. Moreover, the expression levels of IGF- II in tumor tissues are higher than those of surrounding non-tumor tissues in surgical specimens from HBL patients due to biallelic expression of the gene by loss of the methylated status of the promoter^[4]. These facts indicate that IGF- II is deeply involved in the carcinogenesis and progression of HBL.

IGF- II binds to insulin receptor (IR), IGF-I receptor (IGF-IR), and IGF- II receptor^[5]. With the binding of IGF- II, IR mediates glucose metabolism, such as insulin. IGF- II R mediates the degradation of IGF- II, acting as a tumor-suppressor gene. It was expected that IGF-IR mediated the stimulation of proliferation by IGF- II. Indeed, antibody to IGF-IR successfully suppressed the proliferation of HepG2^[6]. Once IGF- II binds to IGF-IR, the receptor autophosphorylates and activates phosphatidyl-inositol (PI) 3-kinase or mitogen-activated protein (MAP) kinase. Further down-stream pathways modulate the gene expression to perform the role of IGF- II.

However, it is not clear whether IGF- II promotes the cell proliferation of HBL, or the signaling pathway of IGF- II to mediate the stimulation of cell proliferation.

Here, we tried to address the possibility of IGF-II in HBL as a growth factor. We also tried to clarify its signaling pathway with inhibitors to signaling pathways, pursuing a potential application for a molecular therapy.

MATERIALS AND METHODS

Cell culture

Huh-6 and HepG2 hepatoblastoma cell lines, were purchased from RIKEN Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's Minimum Essential Medium (Sigma, St. Louis, MO) supplemented with 100 g/L fetal bovine serum (FBS) (Trace Scientific, Melbourne, Australia) in 50 mL/L at 37°C in a humidified chamber. Both cell lines are IGF-IR positive^[7]. For hematoxylin-eosin (H&E) staining, cells were spread onto chamber slides.

Cell viability assay

Freshly thawed cells were seeded onto 10 cm dishes (Asahi Techno Glass, Funabashi, Japan). When they reached sub-confluence, they were trypsinized, harvested, and spread onto 96-well flat-bottom plates with (Asahi Techno Glass) at a density of 1000 cells per well. Following 24 h of culture under DMEM with 100 g/L FBS, medium was changed to DMEM without FBS to quench the FBS effects. After 24 h of culture under DMEM without FBS, IGF-II (Wako Pure Chemicals, Osaka, Japan) was added to the medium. Seventy-two hours later, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay was performed according to the manufacturer's instructions (Promega Corporation, Tokyo, Japan). MTS is bioreduced by cells into a colored formazan product that reduces absorbance at 490 nm. The absorbance was analyzed with a multiple plate reader at a wavelength of 490 nm with a BIO-RAD Model 550 microplate reader (Bio-RAD, Hercules, CA). When LY290042 (Wako Pure Chemicals), PD98059 (Wako Pure Chemicals), Wortmannin (Wako Pure Chemicals), and picropodophyllin (PPP) (Wako Pure Chemicals) were used, each was added to the medium 30 min prior to the addition of IGF-II.

Western blot analysis

Twenty microgram of protein isolated from cultured cells was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis, and transferred to a nylon filter. Primary antibodies were polyclonal rabbit anti-IGF-IR antibody (Cell Signaling Technology, Danvers, MA) and mouse monoclonal anti-Tubulin- α antibody (Lab Vision, Fremont, CA). Second antibodies were horseradish peroxidase (HRP)-linked anti-rabbit antibody (Amersham Bioscience, Tokyo, Japan) and HRP-linked anti-mouse antibody (Amersham Bioscience). Dilutions were 1:500 for primary antibodies, and 1:1000 for second antibodies. The filter was reprobated with anti-Tubulin- α antibody. The specific antigen-antibody complexes were visualized by enhanced chemiluminescence (GE Healthcare Bio-Sciences Corp, Piscataway, NJ).

Statistical analysis

Cell proliferation, demonstrated by MTS assay, was

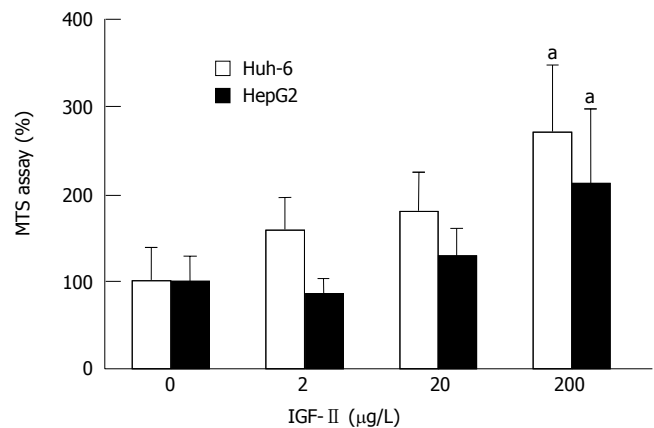


Figure 1 IGF-II stimulated proliferation of hepatoblastoma cells. IGF-II was added to the medium without serum, followed by MTS assay, a modified method of MTT assay (^a $P < 0.05$).

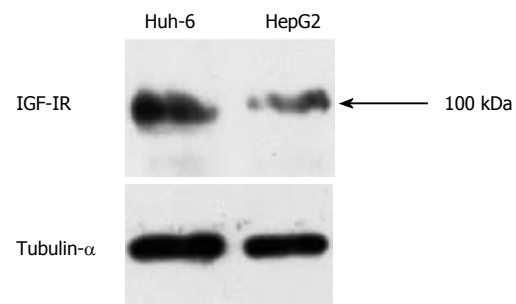


Figure 2 Western blot analysis clearly shows specific bands to IGF-IR. Protein was isolated 72 h after stimulation with IGF-II (200 µg/L). The same membrane was reprobated with anti-Tubulin- α antibody to confirm an equal amount of protein loadings.

analyzed statistically by one-factor analysis of variance. Statistical analysis was performed with JMP5.0J (SAS Institute Japan, Tokyo, Japan). $P < 0.05$ was accepted as statistically significant.

RESULTS

IGF-II stimulated proliferation

Huh-6 and HepG2 proliferated as the concentration of IGF-II increased (Figure 1). When IGF-II was 200 µg/L, cell proliferation of Huh-6 and HepG2 were 2.7 times ($269\% \pm 76\%$) (mean \pm SD) and 2.1 times ($211\% \pm 85\%$) higher than those at 0 µg/L of IGF-II, respectively ($P < 0.05$, $n = 3$).

IGF-IR expressed in Huh-6 and HepG2

Western blot analysis was performed to analyze expression of IGF-IR in Huh-6 and HepG2 since IGF-IR mediates proliferation activity of IGF-II. Protein was isolated from Huh-6 and HepG2 72 h after stimulation with IGF-II (200 µg/L). IGF-IR was expressed in Huh-6 and HepG2 (Figure 2). Tubulin- α was expressed to confirm that equal amount of protein was loaded (Figure 2).

PPP suppressed the stimulation of IGF-II

PPP, a selective inhibitor of IGF-IR, was used to show

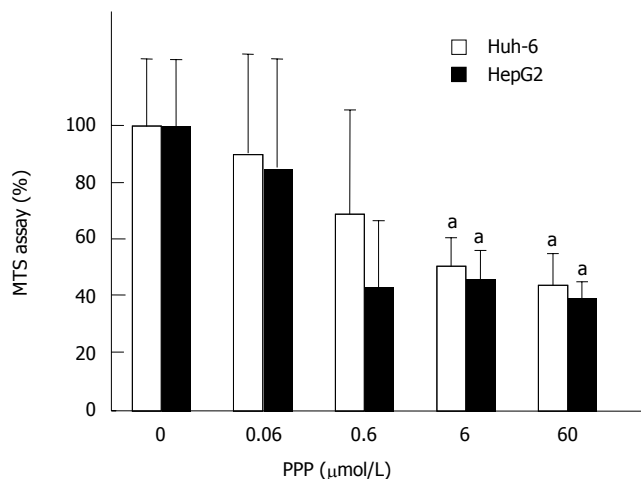


Figure 3 PPP was added to the medium 30 min prior to the stimulation with IGF-II (200 μg/L) and suppressed proliferation of Huh-6 and HepG2 (^a $P < 0.05$).

that IGF-IR mediated the signal of IGF-II (Figure 3). PPP at 60 μmol/L suppressed the cell number of Huh-6 and HepG2 stimulated with 200 μg/L of IGF-II to 44% ± 10% and 39% ± 5%, respectively ($P < 0.05$, $n = 3$).

Stimulation of IGF-II was suppressed by LY294002 and Wortmannin

LY294002 and Wortmannin, specific inhibitors of PI3 kinase, were used to reveal that PI3 kinase mediated the signal of IGF-II (Figure 4). LY294002 at 50 μmol/L and Wortmannin at 200 μmol/L suppressed the cell numbers of Huh-6 and HepG2 stimulated with 200 μg/L of IGF-II to 30% ± 5% and 44% ± 0.4% (Figure 4A) ($P < 0.05$, $n = 3$), and 49% ± 1.0% and 46% ± 1.1% (Figure 4B) ($P < 0.05$, $n = 3$), respectively. PD98059, a specific inhibitor of MAP kinase, was used to clarify whether MAP kinase mediated the signal of IGF-II. PD98059 did not suppress the proliferation of Huh-6 even at 20 μmol/L, while it suppressed that of HepG2 to 33% ± 11%, which was statistically significant ($P < 0.05$, $n = 3$) (Figure 4C).

Suppression of cell proliferation was due to apoptosis

Cultured cells were HE stained to analyze the morphological change 72 h after addition of inhibitors, Huh-6 with PPP, LY294002, and Wortmannin but not with PD98059 while HepG2 with PPP, LY294002, Wortmannin, and PD98059 (Figure 5). Most of the dead cells had pyknotic or fragmented nuclei, indicating apoptosis.

DISCUSSION

The existence of a growth factor in HBL has not been confirmed. It is reported that the expression of IGF-II is elevated in tumor tissues and serum of HBL patients, but its exact role is not clear in terms of carcinogenesis^[8]. In this study, we demonstrated that IGF-II stimulated the proliferation of HBL cell lines. A previous report showed that IGF-II does not stimulate the proliferation of HepG2^[6]. They added IGF-II at a concentration of 200 μg/L as well as fetal bovine serum. We added 200 μg/L of IGF-II to the medium deprived of serum. Serum

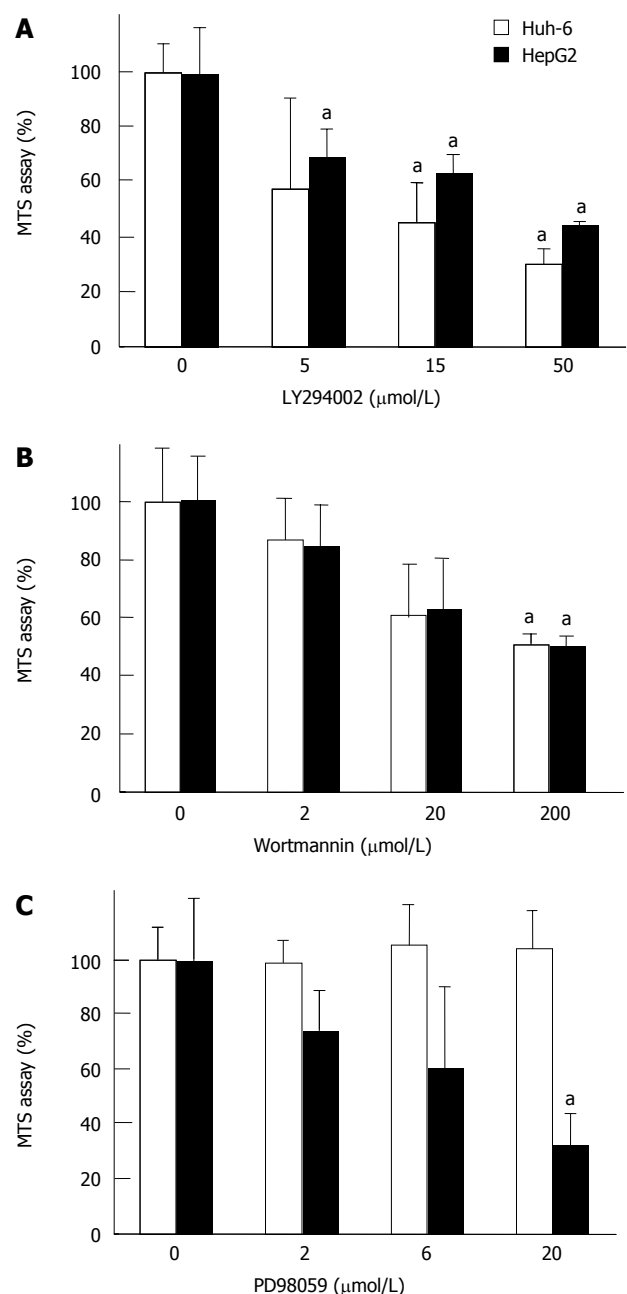


Figure 4 LY294002 or Wortmannin, selective inhibitors of PI3 kinase, or PD98059, a selective inhibitor of MAP kinase, was added to the medium 30 min prior to the stimulation with IGF-II (200 μg/L). LY294002 (A) and Wortmannin (B) suppressed the proliferation of Huh-6 and HepG while PD98059 suppressed HepG2 (C) (^a $P < 0.05$).

stimulates the proliferation of HepG2 to obscure the effect of IGF-II. Moreover, we analyzed Huh-6, another human hepatoblastoma cell line, and revealed that IGF-II stimulated the proliferation of Huh-6^[9]. Our data clearly demonstrated that IGF-II stimulated the proliferation of hepatoblastoma cell lines. Interestingly, HepG2 produces IGF-II and antisense oligonucleotides of IGF-II suppress the proliferation. It may be safe to conclude that IGF-II acts as a growth factor for HBL by autocrine action^[6,10].

The previous report suggested that IGF-II stimulates proliferation *via* IGF-IR^[6]. Our data clearly showed that IGF-IR was expressed in Huh-6 and HepG2. Since IGF-IR mediates proliferation activity of IGF-II, it was expected

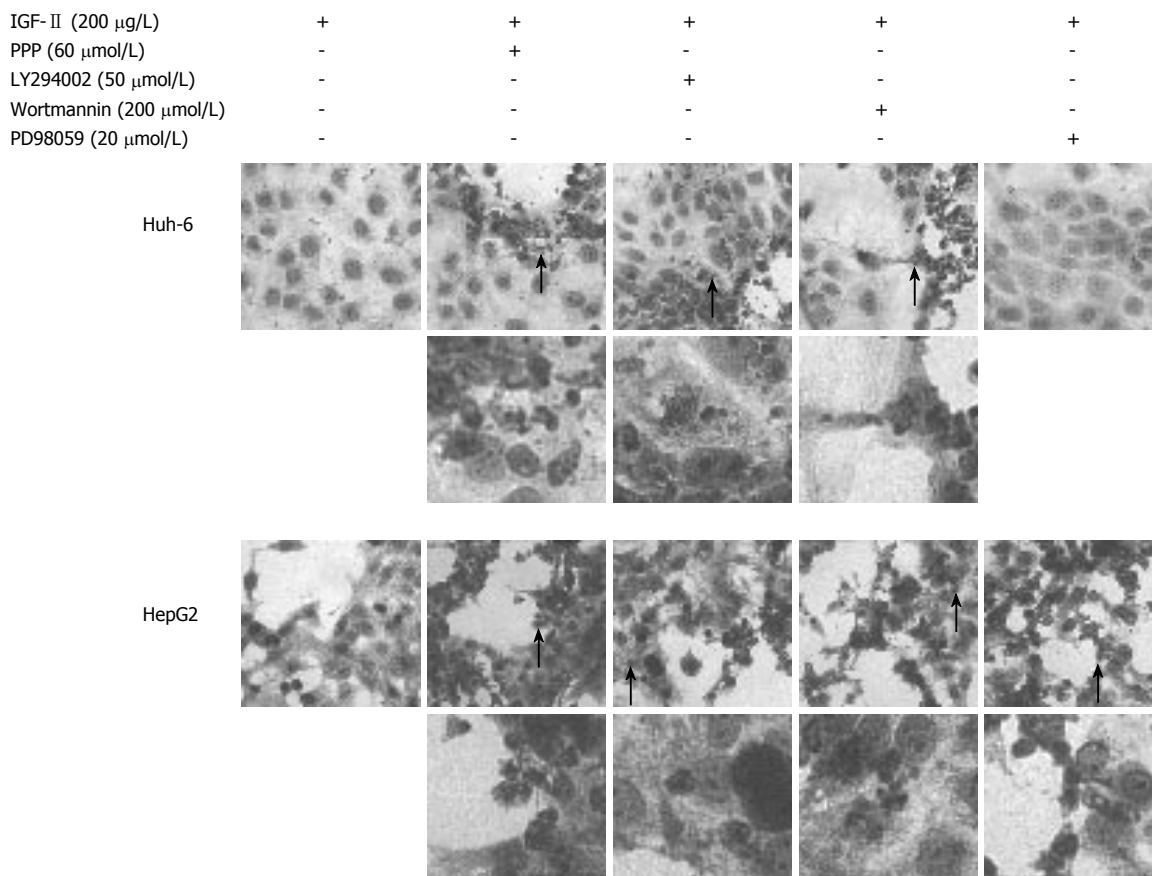


Figure 5 Huh-6 and HepG2 dead due to apoptosis. HE staining was performed to analyze morphological changes after addition of inhibitors. Many Huh-6 cells were dead treated with PPP (60 µmol/L), LY294002 (50 µmol/L), or Wortmannin (200 µmol/L) while HepG2 with PPP, LY294002, Wortmannin, or PD98059 (20 µmol/L). Most of the dead cells had pyknotic or fragmented nuclei (arrows), suggesting apoptosis. Areas indicated by arrows were magnified (x 400).

that an inhibitor of IGF-IR suppressed proliferation^[5]. NVP-AEW541, a tyrosine kinase inhibitor, suppresses the proliferation of HepG2^[7]. We used the commercially available PPP, another tyrosine kinase inhibitor, and it successfully suppressed the proliferation of Huh-6 and HepG2^[11]. Our data on HepG2 was consistent with that of the previous report, and thus was strong evidence that IGF-II mediated the signaling pathway of IGF-II. Since IGF-IR is not involved in glucose metabolism, an inhibitor of IGF-II would be a good candidate for molecular therapy for hepatoblastoma.

PI3 kinase and MAP kinase are the main downstream molecules of IGF-IR^[5]. It was reported that the proliferation of HepG2 cells is suppressed by LY294002 and PD98059^[12], and our results confirmed this. Our results also showed that LY294002 suppressed the proliferation of Huh-6, but PD98059 did not. One may speculate that the stimulation of IGF-II was transmitted *via* PI3 kinase and MAP kinase in HepG2, but only *via* PI3 kinase in Huh-6. Wortmannin, another inhibitor of PI3 kinase, suppressed the proliferation of Huh-6 and HepG2 stimulated by IGF-II, confirming the results of LY294002.

MTS assay measured cell viability, representing cell proliferation. The mechanism of suppression of cell proliferation, however, was not known. To unveil the mechanism, morphological analysis was performed with

HE staining. Many of Huh-6 cells were dead with PPP, LY294002, and Wortmannin while HepG2 with PPP, LY294002, Wortmannin, and PD98059. Nuclei of dead cells were pyknotic or fragmented, indicating apoptosis. It is reported that DNA ladder formation is observed when HepG2 is treated with LY294002, Wortmannin, or PD98059^[5]. It was suggested that Huh-6 and HepG2 were dead due to apoptosis when IGF-I signaling pathway was inhibited. It may be concluded that PI3 kinase would be a better target than MAP kinase for the molecular therapy of hepatoblastoma.

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RAPID COMMUNICATION

A *p53* genetic polymorphism of gastric cancer: Difference between early gastric cancer and advanced gastric cancer

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Abstract

AIM: To investigate the role of the polymorphism of *p53* codon 72 in early gastric cancer (EGC) and advanced gastric cancer (AGC) in Korean patients.

METHODS: DNA was extracted from blood samples of gastric cancer patients ($n = 291$) and controls ($n = 216$). In the *p53* codon 72 genotypes were determined by PCR-RFLP.

RESULTS: Patients with gastric cancer had a significantly higher frequency of the homozygous proline (Pro) allele than the control ($P = 0.032$). Patients with AGC had a significantly higher frequency of the *Arg/Arg* (arginine) allele ($P = 0.038$) than EGC and a similar *Pro/Pro* allele. The signet ring cell type had a higher frequency of the *Pro/Pro* allele than other types ($P = 0.031$). The *Pro/Pro* genotype carries a 3.9-fold increased risk of developing gastric cancer (95% CI, 1.3-15.4, $P = 0.039$) when compared to *Arg/Arg* and *Arg/Pro* genotypes and to develop EGC is a 5.25 fold increased risk (95% CI, 1.8-19.6, $P = 0.021$).

CONCLUSION: The *Pro/Pro* genotype of the *p53* codon 72 polymorphism carries a higher risk for gastric cancer in general and is also associated with a much higher risk for EGC than AGC.

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Key words: *p53* gene; Polymorphism; Gastric cancer

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INTRODUCTION

Gastric cancer is one of the most common malignancies worldwide, although the overall incidence of gastric cancer has been decreasing over the past few decades. Chronic *H pylori* infection and dietary factors, such as those high in salt or nitrate, and nutritional deficiencies have been associated with gastric cancer^[1]. Gastric carcinogenesis is a complex, multistep, and multifactorial process, in which many factors are implicated. The majority of gastric cancers are thought to be caused by environmental factors that result in damage to the mucosa and that inhibit its ability to repair itself^[2]. This response is regulated, in part, by inhibitory and stimulatory factors that are products of proto-oncogenes and tumor suppressor genes^[3].

TP53 gene, an important tumor suppressor gene, encoded *p53* protein. The *p53* tumor suppressor protein was initially isolated in 1979 as a 53 kDa protein that was associated with SV 40 large T antigen^[4]. It was a decade before *p53* was recognized as an important tumor suppressor because of its frequent mutation in human cancers. A large number of human cancers show the evidence of inactivation of the *p53* pathway, suggesting that malignant transformation requires reduction or elimination of *p53*'s function as "guardian of the genome". It is estimated that up to 50% of human cancers carry a mutation of the *p53* gene^[5,6].

Germ line polymorphism of genes involved in multiple steps of carcinogenesis may also account for genetic difference in stomach cancer susceptibility. The *p53* gene is the most intensively studied human gene because of its role as a central tumor suppressor, and has been widely studied in gastric cancer. However, although more than 75% of gastric cancer showed *p53* overexpression, less than 30% had mutation in this gene^[7]. The codon 72 polymorphism is located in exon 4 of the *p53* gene, a region involving very few mutations^[8]. At least two forms of wild-type *p53* protein exist among major human populations; these forms are ascribed to amino acid replacement at codon 72 of *Arg* (CGC) by *Pro* (CCC) in the domain of transactivation of the *p53* protein, though the functional difference between them is unknown. *Pro* variant allele of this *p53* polymorphism has been studied as a potential risk factor for cancer of the lung, breast,

and large bowel, with inconsistent results. Shepherd *et al*^[8] examined the relationship between codon 72 polymorphism and their susceptibilities to gastric cancer in a group of American gastric cancer patients.

In this study, we examined the genotypic frequency of codon 72 in early gastric cancer (EGC) and advanced gastric cancer (AGC) in 292 Korean patients to investigate the role of the *p53* polymorphism.

MATERIALS AND METHODS

Patients

Two hundred twenty two diagnosed gastric cancers were recruited from Ewha Womans University Mokdong Hospital from 2001 to 2005. Their mean age was 56 years (range, 26-88 years); 171 were males and 121 were females. Of the 292 patients, 189 (64.7%) showed advanced gastric cancer, 103 (35.3%) showed early gastric cancer. Two hundred sixteen controls were randomly selected from subjects attending routine medical check-ups (mean age 58 years; range 24-85 years; 128 males and 88 females) who were not affected with stomach cancer by endoscopy. Their details are presented in Table 1. Blood specimens, including serum, plasma, and white blood cells, from the study subjects were also obtained and frozen at -70°C for subsequent analysis. Informed written consent was obtained from all the enrolled patients.

DNA extraction

Genomic DNAs were isolated from peripheral whole blood, 200 μ L (which has been treated with EDTA) by a DNA purifying kit (QIAamp DNA kit Blood Mini Kit, Qiagen, Germany, Hilden) according to the manufacture's instructions.

p53 codon 72 polymorphism

Genotyping of *p53* at codon 72 in exon 4 [M22884. Human phosphoprotein (gi:189467)] was carried out by a polymerase chain reaction (PCR) amplification procedure using primers (*p53*-S: 5'-ATC TAC AGT CCC CCT TGC CG-3 and *p53*-AS: 5'-GCA ACT GAC CGT GCA AGT CA-3'). The amplification reaction was performed in a 6 μ L (0.1 μ g/ μ L) genomic DNA template, 0.1 μ L (10 nmol/mL) of each primer, 1.6 μ L (5 mmol/L per mL) dNTP, 0.1 μ L (0.5 U/ μ L) Taq polymerase (Promega, Madison, WI, USA), and 2 μ L of 10X reaction buffer (200 mmol/L per mL Tris-HCL (pH8.3), 500 mmol/L per mL KCL, and 30 mmol/L per mL MgCl₂). PCR was carried out by 30 cycles under the following conditions: 1 min at 95°C for denaturation, 1 min at 62°C for primer annealing, and 1 min at 72°C for primer extension, using the GeneAmp PCR System 9600 (Applied Biosystem, Foster City, CA). The PCR product was visualized on a 2% agarose gel by electrophoresis, followed by ethidium bromide staining. This generates a 296-base pair fragment. The restriction enzyme BstUI (10 unit, New England Biolabs, Beverly, MA) digests (for 3 h at 60°C) within the sequence corresponding to the *Arg* codon (CGC) at position 72 to generate two visible fragments of 169 bp and 127 bp and leaves the *Pro* allele uncut (Figure 1).

Table 1 Demographic characteristics of patients with stomach cancer (*n* = 292)

	<i>n</i>
Median age	56 (22-88)
Gender	
Male	171
Female	121
Type of stomach cancer	
Early cancer (EGC)	103
Advanced cancer (AGC)	189
Codon 72 polymorphism	
Arg/Arg	101
Arg/Pro	126
Pro/Pro	65
Laurence classification	
Intestinal type	140
Diffuse type	152
Differentiation	
Well/Moderate	114
Poor	132
Signet ring cell	46

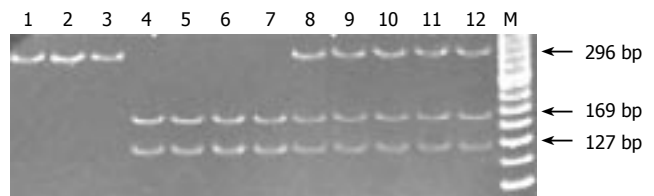


Figure 1 Restriction fragment length polymorphism of PCR-amplified fragment by BstUI. The Lane 1-3 showed only a single undigested band at 296 bp (homozygote of *Pro*), the fragment from lane 4-7 gave two bands at 169 and 127 bp (homozygote of *Arg*), while the fragment from lane 8-12 showed three bands at 296, 169 and 127 bp (heterozygote of *Arg/Pro*). M was a DNA marker.

Statistical analysis

Frequency tables were constructed using the SPSS (11.0 version) statistical package with statistical significance using the χ^2 test. The odd ratios and 95% Confidence interval (CI) were calculated as an approximation of relative risk and adjusted for confounding factors such as age and gender using a logistic regression model.

RESULTS

Distribution of the three genotypes of the *p53* gene

We determined the frequency of the three phenotypes of the *p53* gene in the patients with stomach cancer and controls (Table 2). Genotypes *Arg/Arg*, *Arg/Pro*, and *Pro/Pro* were found 41.2%, 47.7%, and 11.1% in individual controls and 34.5%, 43.1%, and 22.3% in the patients of stomach cancer, respectively. Distribution patterns of the germ line *p53* polymorphism of the patients with stomach cancer included EGC and AGC showed in Table 2. We observed a dramatically increased frequency of *Pro/Pro* allelotype in stomach cancer patient, especially in the patients of EGC.

A logistic regression analysis suggests that the homozygous 72 *Pro* genotype carries a 3.9-fold increased risk of developing gastric cancer (95% CI, 1.3-15.4, *P* =

Table 2 Frequency of the codon 72 genotype

	<i>n</i>	<i>Arg/Arg</i> (%)	<i>Arg/Pro</i> (%)	<i>Pro/Pro</i> (%)
Control	216	89/216 (41.2)	103/216 (47.7)	24/216 (11.1)
Stomach cancer	292	101/292 (34.5)	126/292 (43.1)	65/292 (22.3) ^{a,1}
AGC	189	75/189 (39.7) ^d	76/189 (39.9)	38/189 (20.4) ^b
EGC	103	26/103 (25.8)	50/103 (48.1)	27/103 (26.1) ^{c,2}

^a*P* = 0.032, control *vs* stomach cancer; ^b*P* = 0.029, control *vs* AGC; ^c*P* = 0.027, control *vs* EGC; ^d*P* = 0.038, AGC *vs* EGC; ¹OR = 3.9 (95% CI, 1.3-15.4, *P* = 0.039); ²OR = 5.25 (95% CI, 1.8-19.6, *P* = 0.021); OR: Odd ratio; CI: Confidence interval.

0.039) when compared to *Arg* homozygous and *Arg/Pro* heterozygous. The risk for 72 *Pro* homozygous patients to develop early gastric cancer is 5.35 (95% CI, 1.8-19.6, *P* = 0.021).

p53 polymorphism and histology grading of stomach cancer

We examined the frequency difference in each genotype of *p53* by histological type of all stomach cancers. Germ line *p53* polymorphism was associated with stomach cancer, especially the signet ring cell type of adenocarcinoma (*P* = 0.031, Table 2). There was no relationship between patient gender, tumor stage, the depth of invasion in the wall, histologic type of cancer (Laurence classification: intestinal and diffuse types) and the distribution of codon 72 genotypes.

DISCUSSION

The identification of genes involved in cancer development is critical for uncovering the molecular basis of cancer. The *p53* tumor suppressor protein is essential in the control of cell growth, apoptosis and the maintenance of genomic stability. Loss of *p53* function caused by genomic alterations or interaction with environmental and bacterial products has been suggested as a critical step in multistage human carcinogenesis^[9].

The *p53* gene consists of 11 exons; exons 2-11 code for the protein of 393 aminoacids. The majority of *p53* mutations identified have been found in exons 5-8. However, mutations outside exons 5-8 may occur and they were chiefly observed in exons 4 and 10^[10,11]. At least 10 different polymorphisms have been detected in the human genomic *p53*^[12]. The functional significance of these polymorphisms is currently unknown. The hypothesized relationship between the codon 72 *p53* polymorphism and cancer susceptibility dose not have any mechanistic basis. The *Pro* variant allele of the *p53* polymorphism at codon 72 may not directly affect the *p53* function. It may be in linkage disequilibrium with an as-yet-unidentified functional polymorphism. However, the single-codon difference of the *p53* gene has been demonstrated to result in structurally different proteins^[13]. The polymorphism is localized within a region of polypeptide that was lacking in a deletion mutant of mouse *p53* that had an enhanced ability to immortalize primary rat cells^[14,15].

In a literature review, there was little report about investigation of *p53* gene polymorphism between AGC

Table 3 Distribution of *p53* genotypes and histopathological classification among the patients with gastric cancer

Histopathological classification (<i>n</i>)	<i>p53</i> genotypes		
	<i>Arg/Arg</i>	<i>Arg/Pro</i>	<i>Pro/Pro</i>
Control (216)	89/216(41.2)	103/216(47.7)	24/216(11.1)
Differentiation			
Well to poorly (246)	88/292 (30.1)	151/292 (51.7)	53/292 (18.2)
Signet ring cell (46)	17/46 (37.0)	11/46 (23.0)	18/46 (39.1) ^{a,b}
Tumor histologic type			
Intestinal type (140)	48/140 (34.2)	60/140 (42.8)	31/140 (22.1)
Diffuse type (152)	53/152 (34.8)	66/152 (43.4)	34/152 (22.4)

^a*P* = 0.031, Well to poorly differentiation *vs* Signet ring cell; ^b*P* = 0.019, Control *vs* Signet ring cell.

and EGC comparing with control. We have observed that TP53 codon 72 genotype in stomach cancer and control subjects. Our data further suggest that a *p53* genetic polymorphism was associated with the susceptibility for stomach cancer, especially advanced stomach cancer.

There were several studies conducted to investigate the association between the codon 72 *p53* polymorphism and lung cancer or gastric cancer. The risk increased approximately twofold for smoking-related lung cancer among individuals carrying the *Pro/Pro* genotype compared with those with other genotypes of the codon 72 *p53* polymorphism^[16-18]. A recent study in the patients of gastric cancer showed the significant difference from healthy control, with 48.6% *Arg/Arg* and 3.6% *Pro/Pro* in gastric cancer patients compared with 41.5% and 10.9% in healthy controls^[19]. They showed an increased frequency of *Arg/Arg* genotype in cancer patients at age 75 or more than at a younger age. They suggested the prognosis in patients with *Pro* allele (proline homozygote or *Pro/Arg* heterozygotes) was worse than that those with *Arg/Arg* genotype. They also showed that preferential frequency of codon 72 *Arg p53* acts as a survival factor in gastric cancer patients who have homozygous *Arg* alleles which confer a late start of gastric cancer when compared with those with the *Pro* allele.

In this study we found that the distribution of genotypes had significant difference between the patients of stomach cancer and controls, with 34.5% *Arg/Arg* and 22.3% *Pro/Pro* in stomach cancer patients compared with 41.2% and 11.1% in controls (*P* = 0.032). We observed a significantly higher distribution of *Pro/Pro* genotype in the patient with stomach cancer, especially in the patients with EGC than control. Also in comparison between AGC and EGC, the frequency of *Arg/Arg* genotype was higher than the frequency of AGC, statistically (*P* = 0.038).

The reason for the tissue-specific difference of the germ line *p53* polymorphism was unknown, though an association with histopathologic grading was suggested for gastric cancer^[16]. In this study, we divided two groups whether signet ring cell type or not. Generally, we knew that the stomach cancer with signet ring type was highly malignant and had worse prognosis than other cell types. There was statistically significant difference that the patients with signet ring cell type had a higher ratio of *Pro/Pro* genotype than non-signet ring cell type (*P* = 0.031, Table 3). So the association with histopathologic grading

may suggest that germ line *p53* polymorphism is involved in survival as a clinical prognostic factor as well as cancer susceptibility. Further studies are needed to examine this possibility.

There were several explanations about the role of different genotypes. First, transcriptional properties are different. It has been shown that the *Arg/Arg* and *Pro/Pro* variants differ in binding activity at transcription, to activate transcription, and to induce apoptosis or cell cycle arrest^[20]. The *p53 Arg/Arg* variant induces apoptosis with faster kinetics more efficiently than the *p53 Pro/Pro* variant and the *p53 Arg/Arg* variant is a better inducer of transcription^[19]. Namely, if there was a high proportion of *Arg/Arg* genotype in stomach cancer, it induced the efficient apoptosis or cell cycle arrest, and then it could be better prognosis. Second, wild-type *p53* protein is rapidly degraded, and has a short half-life and low intracellular levels. Stabilization of the protein following an appropriate stimulus such as DNA damage is a physical regulation to increase function^[6]. Different structure of *p53* proteins resulting from a substitution of *Pro* for *Arg* at codon 72 may have different functions in the responsiveness to different stimuli caused by diverse carcinogens such as *H pylori*, dietary, or nutritional deficiency. The reaction of *Pro* genotype and various carcinogens may have more carcinogenic properties. *H pylori* is the well-known cause of chronic gastritis, gastro-duodenal ulcer, and gastric cancer. Some reports studied the relationship between *p53* polymorphism and *H pylori* and found the genotypic frequency of *p53* was similar between cases and controls^[21,22]. We cannot confirm results of *H pylori* status of all patients with stomach cancer in this study, we cannot represent the relationship between *H pylori* and *p53* polymorphism.

In conclusion, the *Pro/Pro* genotype of the *p53* codon 72 polymorphism carries a higher risk for gastric cancer in general (3.9-fold) and is also associated with a higher risk for EGC (5.25-fold) than AGC. Although this finding is provocative, it should be considered preliminary because of the limited sample size. Clearly, a well-designed follow-up study with a larger number of samples is needed to confirm these findings.

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RAPID COMMUNICATION

Molecular analysis of hepatitis B virus isolates in Mexico: Predominant circulation of hepatitis B virus genotype H

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mutations is observed in HBV Mexican isolates.

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Abstract

AIM: To determine the genotypes in Mexican hepatitis B virus (HBV) isolates and characterize their precore and core promoter mutations.

METHODS: Forty-nine HBV isolates of Mexico obtained from sera of 15 hepatitis patients, 6 hemodialysis patients, 20 men seeking HIV testing, and 8 AIDS patients were analyzed. HBV isolates were amplified by PCR, and genotyped by line probe assay (INNO-LiPA HBV Genotyping; INNOGENETICS N V, Ghent, Belgium). HBV genotype confirmation was performed by DNA sequencing part of the sAg region. Precore and core promoter mutation characterization was performed by line probe assay (INNO-LiPA HBV PreCore; INNOGENETICS N V, Ghent, Belgium).

RESULTS: Overall, HBV genotype H was found in 37 (75.5%) out of the 49 isolates studied. HBV genotypes G, A, and D were found in 5 (10.2%), 4 (8.2%), and 3 (6.1%) isolates, respectively. HBV genotype H was predominant in isolates from hemodialysis patients (100%), hepatitis patients (80%), and men seeking HIV testing (75%), and accounted for half of infections in AIDS patients (50%). Six (12.2%) out of the 49 HBV isolates showed both wild type and mutant populations at precore codon 28. These mixed wild type and precore mutant populations were observed in one HBV genotype A isolate and in all HBV genotype G isolates. A dual variant core promoter mutation was observed in 1 (2%) of the isolates, which was genotype H.

CONCLUSION: HBV genotype H is highly predominant in HBV isolates of Mexico followed by genotypes G, A and D. A low frequency of precore and core promoter

INTRODUCTION

Hepatitis B virus (HBV) is an important cause of morbidity and mortality worldwide. It is estimated that 2 billion people are infected with HBV and 350 million individuals suffer from chronic HBV infection in the world^[1,2]. Chronic HBV infection may lead to hepatic fibrosis, cirrhosis and hepatocellular carcinoma (HCC)^[3]. In addition, HBV infection is the 10th leading cause of death worldwide^[1]. HBV is a hepadnavirus that possesses a double stranded DNA genome^[4]. The genetic organization of HBV has been described elsewhere^[4]. Briefly, HBV genome consists of four partly overlapping open reading frames: *S* that encodes for envelope proteins (HBsAg), *C* for core protein (HBcAg) and e antigen (HBeAg), *P* for polymerase protein and *X* for transcriptional transactivator protein^[4]. Based on sequence divergence of the entire genome of > 8%, HBV genomes have been classified into 8 genotypes designated A to H^[5,6]. The distribution of HBV genotypes is geographically restricted. In the American continent, HBV genotypes A and C are common in the USA^[7,8], while genotype F is predominant in Central American countries^[9]. In Europe, HBV genotype D is prevalent in Mediterranean Europe^[10], while genotype A is frequent in Northwest Europe^[11]. In the African continent, HBV genotype E is common in the Sub-Saharan Africa^[12], and genotype A in South Africa^[13]. In the Asian continent, HBV genotypes B and C predominate in south east countries^[14-16], while genotype D is prevalent in central Asia^[17]. Reports indicate that HBV genotypes are related with the severity of liver disease. HBV genotype C has

been associated with the development of liver cirrhosis and hepatocellular carcinoma^[14,18-21]. In addition, HBV genotypes are related with the response to antiviral therapy. Asian studies have shown that compared to HBV genotype B, genotype C has a lower response to interferon alpha (IFN- α) therapy^[22,23]. A recent European randomized trial showed that patients infected with HBV genotypes A and B respond significantly better to pegylated IFN- α -2b alone or in combination with lamivudine than patients infected with HBV genotypes C and D^[24]. Mutations in the precore and core promoter regions of HBV have been also related with genotypes. Precore and core promoter mutations have been observed mainly in HBV genotypes D and B^[25-27], as well as A and C^[25,28,29], respectively.

Little is known about the molecular epidemiology of HBV in Mexico. A previous study on HBV genotypes published in 1998 showed that HBV genotype F is predominant in Mexico^[30]. However, more recently it was discovered that HBV genotype F is divergent^[31], to the extent that a new genotype is split off from genotype F, this new genotype has been designated as HBV genotype H^[9]. Thus some divergent strains formerly classified as HBV genotype F could now be classified as HBV genotype H. Therefore, in order to obtain an updated classification we sought to determine the HBV genotypes and characterize the precore and core promoter mutations in HBV isolates of Mexico.

MATERIALS AND METHODS

HBV isolates

Forty-nine HBV isolates of Mexico were analyzed. HBV isolates were obtained from serum samples of 15 hepatitis patients, 6 hemodialysis patients, 20 men seeking HIV antibody testing, and 8 AIDS patients. All samples were collected in Mexico City and Guadalajara City; both cities are located in central Mexico. Participants were of Mestizo ethnicity. All samples were HBsAg positive. HBeAg, anti-HBe, and HBV DNA levels were not determined. Men seeking HIV antibody testing came from a high risk population for sexually transmitted infection acquisition. Seropositivity for anti-HBc among them (30.4%) was associated to men who had sex with men exclusively and seropositivity for both HIV and herpes simplex virus type 2^[32].

HBV DNA detection and genotyping

HBV DNA was extracted from 200 μ L of serum by using the high pure PCR template preparation kit (Roche Applied Science, Penzberg, Germany) as recommended by the manufacturer. HBV DNA was amplified with HBV pol gene domain B and C primers (INNO-LiPA HBV DR amplification, INNOGENETICS N. V., Ghent, Belgium) by nested PCR as described in the product insert. Primer sequences were used as previously described^[33]. PCR conditions were as follows: 40 cycles at 94°C for 30 s, at 45°C for 30 s and at 72°C for 30 s. HBV genotyping was performed by INNO-LiPA HBV genotyping (INNOGENETICS N. V., Ghent, Belgium) following the manufacturer's instructions.

Sequence analysis was done directly on the second round PCR products using the big dye terminator V3.1 cycle sequencing kit of Applied Biosystems. Sequencing PCR was performed for 25 cycles (at 95°C for 10 s, at 50°C for 5 s, at 60°C for 4 min) and purified on a sephadex column. Three μ L dextran blue/deionised formamide loading buffer was added to the dried pellet and 1 μ L was then loaded on a 4.5% acrylamide slab gel. After electrophoresis of 14 h on the automated sequencer ABI377, data analysis was done using software sequencer 4.1.2.

HBV precore and core promoter mutation characterization

HBV DNA was additionally amplified with basal core promoter and precore primers (INNO-LiPA HBV PreCore amplification, INNOGENETICS N. V., Ghent, Belgium) by nested PCR as recommended by the manufacturer. Primer sequences were used as previously described^[25]. PCR conditions were as follows: 40 cycles at 94°C for 30 s, at 50°C for 30 s and at 72°C for 30 s. Characterization of precore mutations was performed by INNO-LiPA HBV precore (INNOGENETICS N. V., Ghent, Belgium) according to the manufacturer's instructions. This method could identify nucleotide polymorphism at nt 1762 and nt 1764 in the basal core promoter and at codon 28 in the precore region of HBV^[34].

RESULTS

HBV genotyping by INNO-LiPA

For all the 49 HBV isolates, INNO-LiPA HBV genotyping assay was able to provide a genotype result. HBV genotype H was found in 36 (73.5%) out of the 49 isolates studied, while HBV genotypes G, A, D and F were found in 5 (10.2%), 4 (8.2%), 3 (6.1%) and 1 (2.0%) isolates, respectively.

HBV genotyping by DNA sequencing

Thirty-two out of the 36 isolates of HBV genotype H, all 5 isolates of genotype G, and only 1 isolate of genotype F genotyped by INNO-LiPA were selected for DNA sequencing confirmation. All 32 isolates of genotype H and all 5 isolates of genotype G were confirmed by phylogenetic analysis of the HBsAg sequences. However, sequencing and subsequent phylogenetic analysis of the HBV genotype F showed a genotype H. This was caused by a single nucleotide mismatch with the probe on line 11 of the INNO-LiPA HBV genotyping, abolishing the reaction with this probe. This in turn led to a single reactivity with the genotype F probe on line 15. When we compared the HBV genotype H sequences obtained in this study with other published sequences by means of the phylogenetic program PHYLIP, we found that our sequences formed two separate branches within genotype H (Figure 1).

Thus, the final adjusted prevalences of HBV genotypes were 37 isolates (75.5%) of genotype H, 5 isolates (10.2%) of genotype G, 4 isolates (8.2%) of genotype A, and 3 isolates (6.1%) of genotype D. HBV genotype H was predominant in the hepatitis patients, men seeking HIV

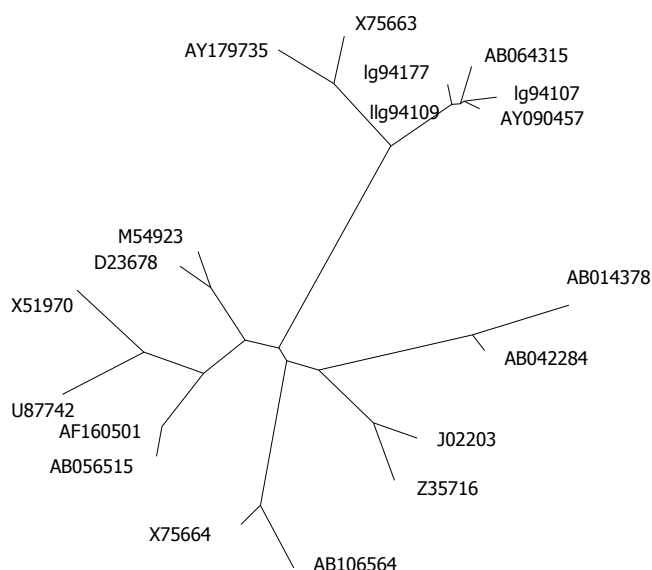


Figure1 Phylogenetic tree constructed with HBV sequences representative of the eight major genotypes, established using neighbor joining based on Kimura 2 parameter method. The three HBV genotype H sequences obtained in this study are shown and have been assigned as IG94107 (Genbank accession number: DQ990454), IG94109 (Genbank accession number: DQ990455) and IG94177 (Genbank accession number: DQ990456).

Table 1 Distribution of HBV genotypes in the populations studied *n* (%)

Group of subjects	<i>n</i>	Genotypes, <i>n</i> (%)			
		A	D	G	H
Hepatitis patients	15	1 (6.7)	2 (13.3)	0	12 (80)
Men seeking HIV testing	20	3 (15)	1 (5)	1 (5)	15 (75)
AIDS Patients	8	0	0	4 (50)	4 (50)
Hemodialysis patients	6	0	0	0	6 (100)
Total	49	4 (8.2)	3 (6.1)	5 (10.2)	37 (75.5)

testing, and hemodialysis patients, and accounted for half of infections in AIDS patients (Table 1). Since the number of HBV isolates analyzed in some groups was very small, we were unable to provide statistical evidence for the distribution of HBV genotypes within the groups.

HBV precore mutations

In the precore region, 41 (83.7%) isolates showed wild type (G) sequence at nucleotide 1896, 6 (12.2%) had a mixture of wild type and G₁₈₉₆A mutation, and 2 had an indeterminate sequence (Table 2). Mixed wild type and precore mutant populations were observed in only one isolate of HBV genotype A but in all isolates of HBV genotype G. Statistical analysis showed that the frequency of mixed wild type and precore mutant populations was significantly higher ($P = 0.04$) in isolates of HBV genotype G than in isolates of HBV genotype A. Mixed wild type and precore mutant populations were observed in 10%

Table 2 Prevalence of precore and core promoter variants and its correlation with genotypes found in Mexican HBV isolates

Region and variant	<i>n</i> (%)	Genotypes			
		A	D	G	H
		(<i>n</i> = 4)	(<i>n</i> = 3)	(<i>n</i> = 5)	(<i>n</i> = 37)
Precore (nt 1896)					
Wild type (G)	41 (83.7)	3	2		36
Wild type + variant (G + A)	6 (12.2)	1		5	
Indeterminate	2 (4.1)		1		1
Core promoter (nt 1762 and 1764)					
Wild type (AG)	44 (89.8)	4	2	5	33
Dual variant (TA)	1 (2)				1
Indeterminate	4 (8.2)		1		3

of isolates from men seeking HIV testing and in 50% of isolates from AIDS patients only. The frequency of these mixed wild type and precore mutant populations in AIDS patients was significantly higher than that in isolates from hepatitis patients ($P = 0.007$), and men seeking HIV testing ($P = 0.03$). This frequency of mixed wild type and precore mutant populations in AIDS patients was also higher but not statistically significant ($P = 0.06$) than that in isolates from hemodialysis patients.

In the core promoter region, 44 (89.8%) isolates had wild type sequence (A at nucleotide 1762 and G at nucleotide 1764), 1 (2%) showed the classical dual variant (A₁₇₆₂T, G₁₇₆₄A), and 4 (8.2%) had an indeterminate sequence. The only one dual variant was found in a sample of a hepatitis patient infected with HBV genotype H. For its part, all 4 samples with indeterminate sequences were found in hepatitis patients too. Three of them were found in isolates of HBV genotype H, and in one isolate of HBV genotype D.

DISCUSSION

In this work, we found a predominant frequency of HBV genotype H in Mexican isolates. The extremely high frequency of this HBV genotype H in Mexico has not been reported before. A previous study in Mexican isolates from blood donors and patients with liver disease showed infections with only 3 HBV genotypes: A, D and F in both populations^[30]. Nevertheless, in the present study we have found 4 HBV genotypes. It was known that HBV genotype F was predominant in Mexico^[30], but also it was observed that the predominant genotype F in Mexico is divergent^[31]. These results contrast with those found in the present study since HBV genotype H was predominant whilst HBV genotype F was not present anymore. Genotype H which was discovered recently is closely related with genotype F, while genotype H is most likely split off from genotype F^[9]. Therefore, a number of HBV isolates previously classified as genotype F are now classified as genotype H. Thus, it explains the remarkable change in HBV genotype distribution in Mexico with a predominant circulation of genotype H. The high

frequency of HBV genotype H in this study is the highest reported worldwide. Indeed, this genotype has been rarely found or not reported at all in most countries^[35-39]. HBV genotype H has been found only in HBV isolates of Nicaragua, Mexico, USA^[9,31,35], and Japan^[37,39]. In our study, HBV genotype H was predominant in the hepatitis patients, men seeking HIV testing, and hemodialysis patients. In addition, genotype H was responsible of 50% of infections in AIDS patients. Reports on genotypes found in hepatitis patients from other countries remark the differences in geographical distribution of HBV genotypes in the world. In the USA and other countries a high frequency of HBV genotype B and C has been found in hepatitis patients^[40,41]. HBV genotypes B and C have been linked to Asian ethnicity^[7,35], and the absence of these genotypes in our populations studied reflects the absence of HBV isolates of Asian origin. Frequencies of HBV genotypes found in men seeking HIV antibody testing are difficult to compare since there are not further reports for the comparison. Concerning hemodialysis patients, HBV genotype H was responsible for all infections in our study. This prevalence is clearly different from those found in hemodialysis patients of other countries. For instance, in a Turkish study all isolates of hemodialysis patients were genotype D^[42], in an Indonesian study all isolates were genotype B^[43], and in a Brazilian study genotypes A and D were the most frequently found^[44]. These HBV genotypes reported in hemodialysis patients certainly reflect the predominant HBV genotypes found in their respective countries. For its part, HBV genotype G is responsible for half of infections in AIDS patients. HBV genotype G has been discovered in USA and France isolates by Stuyver *et al.*^[45]. Interestingly, all HBV genotype G isolates were found as single isolates (no co-infections) in our study. This finding is unexpected, since HBV genotype G is frequently found as a co-infection with HBV genotype A^[46,47]. The HBV genotype frequencies in AIDS patients found in our study differ substantially from those found in a Spanish study where researchers found a predominant HBV genotype A followed by genotype D among the AIDS patients studied^[48]. The low frequency of precore mutants in Mexican isolates found in the present study confirms a previous observation^[30]. In addition, this frequency of precore mutations contrasts with a higher frequency observed in the USA and Hong Kong^[7,34,49]. The distribution of precore mutations in HBV genotypes found in the present study differs substantially from those found in other studies. While other researchers have found precore mutations in HBV genotypes B, C^[50,51], and D^[27], we have found the mutations only in HBV genotypes G and A, being mutations largely predominant in HBV genotype G (all HBV genotype G isolates in this study). The existence of precore mutations in HBV genotype G has been reported in patients chronically infected with HBV^[45]. Remarkably, all mutated HBV genotype G isolates in this study came from samples of AIDS patients and one of the men seeking HIV testing. No similar findings are reported in AIDS patients. Concerning mutations in the core promoter region, it is remarkable that the classical dual variant (Δ ₁₇₆₂T, G₁₇₆₄A), and indeterminate sequences were observed only in hepatitis patients. The prevalence

of core promoter mutations and its distribution in HBV genotypes found in this study also differ from those reported in other countries. A large study with Hong Kong and USA samples showed that the frequency of dual variant is 20% and 30.2%, respectively^[34], which is significantly higher than that found in our study. HBV core promoter mutations have been found in HBV genotypes A, B C and D^[25,29,34]. Nevertheless, these mutations were found mainly in HBV genotype H followed by HBV genotype D in the present study. Indeterminate sequences in precore and core promoter regions as well as the change in genotype F in LiPA to genotype H found in the Mexican isolates are most probably due to mismatching of the sequences with the probes used. A single mismatch is sufficient to abolish hybridization to a probe under the conditions of the assay as previously described^[34].

The INNO-LiPA HBV genotyping and precore assays have proved to be rapid, sensitive and reliable for the detection of HBV genotypes and precore promotor/precore mutations.

The genetic variability of HBV has implications on the sensitivity of immunologic and molecular based assays^[5]. In addition, HBV genotyping has shown its utility not only in epidemiology studies but also in predicting prognosis and therapeutic response^[6,52,53].

In conclusion, HBV circulates in at least 4 different genotypes in Mexico. HBV genotype H is highly predominant in HBV isolates of Mexico followed by genotypes G, A and D. The frequency of precore mutations in Mexican HBV isolates is low and associated mainly with HBV genotype G. Core promoter mutations seem to be rare in HBV isolates of Mexico.

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RAPID COMMUNICATION

Prevalence of celiac disease in an urban area of Brazil with predominantly European ancestry

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the prevalence of biopsy-confirmed celiac disease was approximately 1:417, similar to that seen in European countries.

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Key words: Celiac disease; Epidemiology; Malabsorption; Prevalence; Small bowel disease

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Abstract

AIM: To determine the prevalence of celiac disease in a group of volunteer blood donors at a blood bank in the city of Curitiba, Brazil through detection of the serum marker immunoglobulin A (IgA) antitransglutaminase antibody.

METHODS: Blood samples collected from 2086 healthy subjects at the Paraná State Center for Hematology and Hemotherapy in Curitiba were submitted to ELISA testing for the IgA antitransglutaminase antibody. Positive samples received IgA antiendomysium antibody test through indirect immunofluorescence using human umbilical cord as substrate. Subsequently, patients who were positive on both tests underwent small bowel (distal duodenum) biopsy.

RESULTS: Six subjects, four males and two females, tested positive for the two serum markers. Five of the six were submitted to intestinal biopsy (one declined the procedure). Biopsy results revealed changes in the distal duodenum mucosa (three classified as Marsh IIIb lesions and two as Marsh II lesions). Most donors diagnosed having celiac disease presented multiple symptoms (gastrointestinal tract complaints). One donor reported having a family history of celiac disease (in a niece).

CONCLUSION: Among apparently healthy blood donors,

INTRODUCTION

Celiac disease (CD) is a chronic disease that affects genetically susceptible individuals and is characterized by permanent intolerance to gluten and other related proteins, causing nonspecific, characteristic lesions in the small bowel mucosa^[1]. These lesions prevent nutrient absorption in the affected area. Treatment involves a strict gluten-free diet (elimination of products containing wheat, barley, rye and probably oats). Clinical manifestations of CD are protean in nature and vary markedly with the age of the patient, the duration and extent of disease, and the presence of extraintestinal pathologic conditions. In addition to the classical gastrointestinal form, a variety of other clinical manifestations of the disease have been described, including atypical and asymptomatic forms^[2].

Serologic tests developed over the past decades provide noninvasive tools for screening individuals at risk for the disease in both general and selected populations. However, for diagnosis of CD, it is necessary to perform histological evaluation in order to confirm intestinal damage in individuals presenting positive exam results.

Initially, the disease was considered a low-prevalence disorder and, consequently, diagnosis was based on clinical findings alone. Most prevalence studies of the disease have been carried out in European countries and have evaluated the prevalence based on symptomatic cases. Therefore,

atypical and asymptomatic cases were frequently misdiagnosed. Currently, prevalence is higher due to the availability of reliable, easily administered serologic tests of high sensitivity and specificity.

In CD patients, a wide variety of clinical manifestations are seen, and the disease may be seen in conjunction with other diseases. Therefore, as well as being important tools for prevalence studies, serologic tests (performed prior to the mandatory intestinal biopsy used for diagnosis confirmation) have become important markers for the disease^[3].

Studies carried out in Europe, USA and Brazil in order to investigate the prevalence of CD have shown that the prevalence of the disease is considerably higher than previously presumed. Recent studies of serum markers in blood donors have shown a prevalence of 1 to 250 in Sweden^[4], 1 to 524 in Denmark^[5], 1 to 333 in Holland^[6], 1 to 157 in Israel^[7] and 1 to 250 in the USA^[8]. The first Brazilian epidemiologic study using serum markers was published in 2000 and showed the prevalence of CD to be 1 to 681 among blood donors in the national capital of Brasília^[9].

In order to get further insight into the prevalence of CD in Brazil and to evaluate the influence of genetic and environmental predisposition, we chose the city of Curitiba (in the state of Paraná). The city is located in the southern part of Brazil, and its population is of mostly European ancestry. Eating habits in Curitiba are similar to those seen in the countries of origin, and the diet features a significant wheat component. We decided to evaluate the epidemiology of CD in blood donors at a blood bank and to simultaneously analyze the ethnic profile of these donors. To that end, we used serum markers and conducted a genealogical study.

MATERIALS AND METHODS

Subjects

We analyzed 2086 serum samples collected from healthy donors at the Paraná State Center for Hematology and Hemotherapy in Curitiba from January to December 2001, regardless of gender. Donor ages ranged from 20 to 62 (mean, 33 years). The Ethics Committee of the University of São Paulo School of Medicine Hospital das Clínicas and the Ethics Committee of the Paraná State Center for Hematology and Hemotherapy approved the study. Donors gave written informed consent and answered a standardized questionnaire on demographics and health. No significant disease was diagnosed, and all serologic tests for human immunodeficiency virus (HIV), hepatitis B, hepatitis C, and alanine transaminase were normal. Serum samples were centrifuged and stored at -80°C after serologic tests.

Methods

Serologic tests were performed in three University of São Paulo School of Medicine Medical Investigation Laboratories (LIM), LIM 06, LIM 07 and LIM 56. Samples positive for IgA antitissue transglutaminase (anti-tTG) by enzyme-linked immunosorbent assay (ELISA) were submitted to an immunofluorescence test for IgA antiendomysium

antibody (EMA) using human umbilical cord as substrate. Each patient positive for both IgA anti-tTG and IgA EMA underwent small bowel (distal duodenum) endoscopic biopsy in which four samples were collected. This is the classical procedure accepted by the European Society of Pediatric Gastroenterology and Nutrition (ESPGAN)^[10] for confirmation of a diagnosis of CD.

Anti-tissue transglutaminase antibody test

Technique carried out in accordance with the method described by Dieterich *et al.*^[11]: Microplates (96 wells; Corning, New York, NY, USA) were coated with 1 µg of guinea pig liver tTG (T 5398; Sigma, St. Louis, MO, USA) per well (activity: 1.5-3 KU/g of protein) in 100 µL Tris-HCl (50 mmol/L); NaCl (150 mmol/L) and CaCl₂ (5 mmol/L), pH 7.5, and incubated at 37°C for 2 h. The wells were extensively rinsed in an Immunowash microplate washer (Bio-Rad, Hercules, CA, USA) in Tris-HCl (50 mmol/L), NaCl (150 mmol/L), EDTA (10 mmol/L) and Tween 20 (1 g/L), pH 7.4, and the microplates were incubated in washing buffer (100 µL/well) at 4°C overnight. The microplate solution was aspirated; serum samples were diluted (1/100) in washing buffer, poured into the respective wells (100 µL/well) and incubated for one hour at room temperature. After rinsing the plates (3 times), peroxidase-conjugated IgA-α antibody (A 0295, Sigma), diluted (1/1000) in washing buffer (100 µL/well), was added to the wells, which were then incubated at room temperature for one hour. Extensive rinsing eliminated unbound antibodies. The tetramethylbenzidine substrate (100 µL/well) promoted the color reaction. Microplates were placed in the dark for approximately 10 min, and subsequently blocked with 0.5 mol/L H₂SO₄ (50 µL/well). Absorbance values were read at 450 nm using an ELISA reader (VERSAmix tunable microplate reader; Molecular Devices, Sunnyvale, CA, USA). After subtraction of background values < 0.100, absorbance (A) readings were multiplied by the serum dilution in order to calculate the ELISA titers. With the inclusion of two control serum samples in every test, intra-assay variations (*n* = 22) and inter-assay variations (*n* = 24) were 8.68% and 8.38%, respectively. Statistical evaluation was carried out through receiver operator characteristic (ROC) curve analysis^[12] using SPSS® software.

Antiendomysium antibody test

Immunofluorescence tests for antiendomysium antibodies were carried out using 2 µm cryosections of human umbilical cord, which were incubated with patient serum prediluted (initial dilution = 1:5) in buffer (PBS and 1 g/L Tween 80, pH 7.2), in a humid chamber at 37°C for 30 min. Slides were rinsed twice in PBS, pH 7.2, for 5 min. Samples were then incubated with fluorescein-conjugated anti-human IgA (Sigma) and diluted in dilution buffer (1:30). Subsequently, samples were rinsed twice with PBS and the slides were again incubated in humid chamber at 37°C for 30 min. Later, samples were read under fluorescence microscopy. Samples were considered positive if there was a hexagonal pattern of fluorescence throughout the peritubular muscle layer of the human umbilical cord vessels, marking the extracellular connective tissue.

Table 1 Celiac disease diagnosed in apparently healthy blood donors

Age	Gender	Race	Ancestry	GI symptoms	Anti-tTG ¹	EMA ¹	Histology
26	F	W	Italian and Portuguese	Diarrhea, distension, abdominal pain and flatulence	1025	1/1280	Marsh IIIb
26	F	W	Italian, Portuguese and German	Pyrosis, obstipation, epigastric pain, flatulence, weakness and fatigue	295	1/640	Marsh IIIb
42	M	W	Portuguese, German and Spanish	Pyrosis, obstipation and flatulence	40	1/80	Marsh II
46	M	W	Italian	Pyrosis	2816	1/2560	Marsh II
57	M	W	Italian and Portuguese	Diarrhea	1540	1/640	Marsh IIIb
57	M	W	Italian	Asymptomatic	1840	1/640	No biopsy

F: Female; M: Male; W: White; GI: Gastrointestinal; Anti-tTG: Antitissue transglutaminase; EMA: Antiendomysium antibody; ¹Titers.

Histology

Samples were fixed with buffered formalin and stained with hematoxylin and eosin (H&E) for histological study. The following aspects were evaluated: (1) crypt/villus ratio; (2) crypt regeneration; (3) characteristics of the inflammatory infiltrate in the section itself; (4) type of atrophy. Two pathologists examined every slide for the standardization of the histological aspects, using the histological classification developed in 1992 by Marsh and modified in 1997 by Rostami *et al*.^[13-15] This modified system establishes five lesion classes. In Marsh 0, there is normal architecture of the mucosa and less than 40 intraepithelial lymphocytes per 100 enterocytes in the villus epithelium. Marsh I is defined as normal architecture of the mucosa and more than 40 lymphocytes per 100 enterocytes in the villus epithelium. Marsh II involves crypt enlargement (hyperplasia), in which immature epithelial cells are produced in large numbers and there is an influx of lymphocytes and plasmocytes. Under this system Marsh III has been reclassified and divided into three separate classes. In Marsh IIIa, there is partial villus atrophy combined with slight lymphocyte infiltration in epithelial cells and crypt hyperplasia. Marsh IIIb is marked by near total atrophy of the villi (villi still recognizable), crypt hyperplasia in which immature epithelial cells are produced in greater proportions, and influx of inflammatory cells. The final designation, Marsh IIIc, indicates total villus atrophy, hyperplastic crypts and infiltrative lesions^[13-15].

RESULTS

Of the 2086 blood donors, 1437 (68.88%) were males and 649 (31.12%) were females. Mean age was 33. There were 1977 Whites (94.77%), 82 Blacks (3.93%), and 27 Asians (1.30%). There were 1179 who claimed European ancestry (56.52%). Ethnic data were obtained through a genealogical study of the preceding three generations (Figure 1).

We identified six donors (four males and two females) who were positive for both anti-tTG and EMA. Five of these were submitted to intestinal biopsy and one declined the procedure. The procedure revealed that, in the mucosa of the small bowel (distal duodenum), three of the subjects presented Marsh IIIb lesions and two presented Marsh II lesions. Most subjects diagnosed with CD reported various gastrointestinal symptoms. One subject reported a family history of CD in a first-degree relative (a niece) (Table 1).

The prevalence of biopsy-confirmed CD was approximately 1:417 among apparently healthy blood

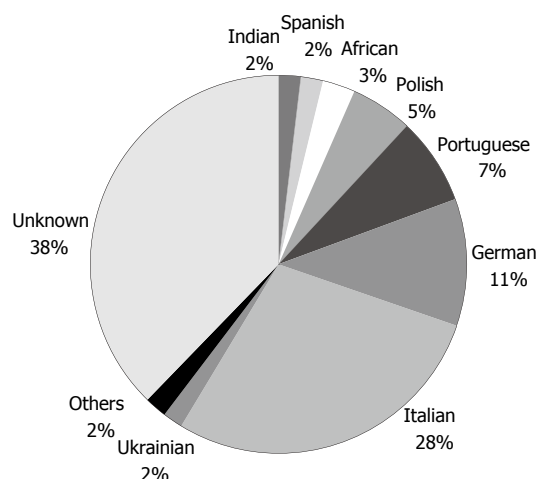


Figure 1 Blood donors by ancestry.

donors. When the cases were positive for antitransglutaminase antibody were confirmed through the use of another marker, antiendomysium antibody, the prevalence was 1:347.

The sensitivity and specificity of the anti-tTG test were 100% and 96%, respectively. The OD cutoff value, established through analysis of the ROC curve, was 0.238. The area of the ROC curve was 0.999 ± 0.002 .

DISCUSSION

In blood donors at a blood bank in Curitiba (Paraná), the prevalence of CD was 1:347 when samples positive for IgA anti-tTG antibodies were tested for a second marker (IgA antiendomysium antibodies). When subjects positive for both serum markers underwent distal duodenum biopsy, the prevalence was 1:417. This high prevalence is similar to that seen in European countries. This is unsurprising since most of the subjects in the study group were of European descent: 56.52% claimed European ancestry, and 37.78% were unaware of their ancestry (Figure 1). We can assume that most of the subjects of unknown ancestry were also of European descent since this ethnic group is predominant in Curitiba.

Factors that influence the high prevalence of CD are the genetic component (HLADQ₂ and DQ₈ haplotypes, which are strongly associated with CD and highly prevalent in the European population) and the consumption of grains containing gluten^[2]. These two factors are present

in the city of Curitiba, where, in recent decades, there has been an increase in the consumption of wheat flour. This increase is related to the lifting of sanctions on the importation of special wheat grains. In addition, the consumption of foods containing wheat has increased as a consequence of economic plans that have created conditions which facilitate consumption among the population^[17]. Currently, wheat consumption in the south of Brazil, where Curitiba is located, is 61 kg/person per year-the highest in the country^[17]. This corresponds to 43.57% of the consumption in Italy, which, according to the World Health Organization, is 140 kg/person per year.

Another relevant and notable aspect is that, of the six positive donors, five were of Italian descent, most from the region of Veneto (northern Italy). A study carried out in that region using serum marker screening showed a CD prevalence of 1:200^[18].

The CD prevalence observed in the present study was higher than the 1:681 prevalence found in a similar study involving blood donors at a blood bank in the city of Brasília^[9]. This low prevalence could be attributed to low numbers of individuals of European descent, although the actual ethnic composition of the Brasília population is not known. However, it is known that most of the population of the city migrated from the Central-West region of Brazil, where individuals of European ancestry are in the minority and the consumption of wheat is lower (23 kg/person per year)^[17].

In the literature, the prevalence of CD is higher in females when studies are based on cases with clinical evidence^[19]. However, recent studies of blood donors showed that, when the diagnosis is made through the use of serum markers, this relationship is not maintained^[7,8]. In our study, approximately one-third of the participants were female, and we found the prevalence of CD to be similar between males and females and proportionate to their respective representation in the study population (four males and two females tested positive).

A prevalence study of CD in a population composed of the descendants of European immigrants to New Zealand was conducted in the city of Wellington^[20]. The study was based on clinical findings in children and adults and showed that prevalences of the disease in children and adults were lower and the same as those seen in the respective European cities of origin. The authors attributed the lower prevalence in children to underdiagnosis. Although the population under study presented the same ancestry and possibly the same eating habits, the authors evaluated only "the tip of the celiac iceberg". In the present study, which was conducted in a region of high wheat consumption and included a genealogical search and the use of serum markers, we evaluated "the rest of the iceberg". Our results show that, when ethnic and environmental factors are constant, the prevalence of the disease remains stable, even if members of the population migrate to other areas. A recent study^[21], conducted in Argentina, using serum markers to evaluate a population of European descent in an area with 85 kg/person per year of wheat consumption, showed a CD prevalence of 1:167, reinforcing our hypothesis.

In the present study, we found a prevalence of CD of 1:417 among apparently healthy blood donors. Our results demonstrate that the high prevalence of CD in the city of Curitiba is comparable to that seen in European countries. This finding supports the hypothesis that the prevalence of the disease remains stable if predisposing genetic and environmental conditions are maintained.

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Endoscopic management of acute cholangitis in elderly patients

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Abstract

AIM: To evaluate clinical presentation, etiology, complications and response to treatment in elderly patients with acute cholangitis.

METHODS: Demographics, etiology of biliary obstruction, clinical features, complications and associated systemic diseases of 175 patients with acute cholangitis were recorded. Endoscopic biliary drainage was performed using nasobiliary drain or stent. The complications related to ERCP, success of biliary drainage, morbidity, mortality and length of hospital stay were evaluated.

RESULTS: Of 175 patients, 52 aged ≥ 60 years (group I, age < 60 years; group II, age ≥ 60 years) and 105 were men. Fever was present in 38 of 52 patients of group II compared to 120 of 123 in group I. High fever (fever $\geq 38.0^{\circ}\text{C}$) was more common in group I (118/120 vs 18/38). Hypotension (5/123 vs 13/52), altered sensorium (3/123 vs 19/52), peritonism (22/123 vs 14/52), renal failure (5/123 vs 14/52) and associated comorbid diseases (4/123 vs 21/52) were more common in group II. Biliopancreatic malignancy was a common cause of biliary obstruction in group II ($n = 34$) and benign diseases in group I ($n = 120$). Indications for biliary drainage were any one of the following either singly or in combination: a fever of $\geq 38.0^{\circ}\text{C}$ ($n = 136$), hypotension ($n = 18$), peritonism ($n = 36$), altered sensorium ($n = 22$), and failure to improve within 72 h of conservative management ($n = 22$). High grade fever was more common indication of biliary drainage in group I and hypotension, altered sensorium, peritonism and failure to improve within 72 h of conservative management were more common indications in group II. Endoscopic biliary drainage was achieved in 172 patients (nasobiliary drain: 56 group I, 24 group II, stent: 64 group I, 28 group II) without any significant age related difference in the success rate. Abdominal

pain, fever, jaundice, hypotension, altered sensorium, peritonism and renal failure improved after median time of 5 d in 120 patients in group I (2-15 d) compared to 10 d in 47 patients of group II (3-20 d). Normalization of leucocyte count was seen after a median time of 7 d (3-20 d) in 120 patients in group I compared to 15 d (5-26 d) in 47 patients in group II. There were no ERCP related complications in either group. Five patients (carcinoma gallbladder $n = 3$, CBD stones $n = 2$) died in group II and they had undergone biliary drainage after failure of response to conservative management for 72 h. There was a higher mortality in patients in group II despite successful biliary drainage (0/120 vs 5/52). Length of hospital stay was longer in group II patients (16.4 ± 5.6 , 7-30 d) than in group I patients (8.2 ± 2.4 , 7-20 d).

CONCLUSION: Elderly patients with acute cholangitis have a high incidence of severe cholangitis, concomitant medical illnesses, hypotension, altered sensorium, peritonism, renal failure and higher mortality even after successful biliary drainage.

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Key words: Acute cholangitis; Endoscopic biliary drainage; Endoscopic retrograde cholangio-pancreatography; Common bile duct stones; Carcinoma gall bladder

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INTRODUCTION

Acute cholangitis is a difficult diagnostic and therapeutic problem. Classically, Charcot's triad of jaundice, abdominal pain and fever have been the main basis of diagnosis however 30%-45% of the patients with acute cholangitis do not satisfy the criteria of Charcot's triad^[1].

Altered sensorium, hypotension and renal failure can often be seen in patients with suppurative cholangitis^[2]. In many cases, bile duct infection is latent and does not cause symptoms. Cholangitis varies in severity from a mild form which responds to parenteral antibiotics alone to severe or suppurative cholangitis which requires early drainage of biliary system to reduce the incidence of

systemic complications^[3]. Endoscopic biliary drainage is an established mode of treatment for acute cholangitis with high success rate, low morbidity and mortality^[1,3-20].

Old age is considered as a risk factor associated with significant mortality in acute cholangitis^[21,22]. It is also a factor which adversely affects the outcome and survival in patients with acute cholangitis, which may be due to associated other systemic diseases and comorbid conditions, underlying etiology of biliary obstruction, poor immune status and poor general health in old age^[21-23]. However, there is no study on clinical presentation, etiology, complications and survival after endoscopic biliary drainage in elderly patients with acute cholangitis. We prospectively compared the clinical presentation, etiology, associated systemic diseases, complications and response to treatment in elderly patients with acute cholangitis.

MATERIALS AND METHODS

Over past 5 years, 175 patients with acute cholangitis were recruited in this study. The diagnosis of acute cholangitis was based on presence of clinical evidence of infection (fever, leucocytosis and abdominal pain) in patients with biliary obstruction in the form of jaundice or hyperbilirubinemia^[1,3]. Patients with biliary obstruction and associated hypotension, impaired level of consciousness and renal failure were specifically looked for associated features of acute cholangitis like fever and leucocytosis. They were labelled to be having acute cholangitis if they had any of features like hypotension, impaired level of consciousness and renal failure along with fever and /or leucocytosis in absence of infection at other sites or other causes for hypotension, impaired level of consciousness and renal failure. Details of demographic data, etiology of biliary obstruction, clinical features, biochemical parameters, microbiological spectrum, complications and associated other systemic diseases were recorded.

Immediate endoscopic biliary drainage was performed in patients with high fever, hypotension, peritonism, altered sensorium and associated renal failure. In patients without these features, endoscopic biliary drainage was performed only if they did not respond to conservative treatment. All patients were treated with intravenous antibiotics and metronidazole. Concomitant medical illnesses were also recorded and treatment was started to control these medical illnesses. Endoscopic retrograde cholangiopancreatography (ERCP) was performed with a side viewing duodenoscope with a large accessory channel in a standard manner. Patients were subjected to either nasobiliary drain (NBD) placement or placement of biliary stent. After cannulation of the common bile duct, bile was aspirated to confirm the position of the cannula. Injection of contrast was avoided as far as possible to prevent any sudden increase in bile duct pressure which could lead to sudden cholangiovenous and cholangiolymphatic reflux. Endoscopic sphincterotomy was not performed. Either a 7-F nasobiliary drain or 7-F straight flap stent was placed in bile duct. After ERCP and biliary drainage, all the patients were kept under strict observation and treated in a critical care ward and the amount of bile drained from

Table 1 Demographic and clinical characteristics of patients with severe acute cholangitis

	Group I	Group II
<i>n</i>	123	52
Mean age \pm SD (range) years	38.6 \pm 12.4 (20-56)	68.4 \pm 10.8 ^a (60-90)
Gender (Male: Female)	72:51	33:19
Clinical presentation <i>n</i> (%)		
Right upper	116 (94.3)	27 (52) ^a
Quadrant pain	118 (96)	18 (34.6) ^a
Fever \geq 38.0°C	2 (1.6)	20 (38.4) ^a
< 38.0°C	120 (97.5)	48 (92.3)
Jaundice	3 (2.4)	19 (36.5) ^a
Altered sensorium	5 (4.0)	13 (25.0) ^a
Hypotension	22 (17.8)	14 (11.3) ^a
Peritonism	5 (4.0)	14 (11.3) ^a
Renal failure	4 (3.2)	21 (40.3) ^a
Other systemic illnesses		

^a*P* < 0.05.

NBD was monitored daily.

The patients were closely observed for evidence of ERCP-related complications. Clinical improvement was defined as normalization of fever, leucocytosis, hypotension, peritonism, altered sensorium and renal functions. After clinical improvement, a repeat ERCP or NBD cholangiogram was obtained in each patient. Clinical monitoring and biochemical tests were performed before ERCP and then on d 1, 3 and 7 after ERCP or earlier if indicated. After improvement of their cholangitis, patients underwent some form of definitive management. Primary outcome measures included complications related to ERCP and the treatment outcomes including success of biliary drainage, morbidity, mortality and length of hospital stay.

Statistical analysis

Quantitative data were expressed as the mean \pm SD or as the median. The Mann-Whitney *U* test was used for comparing continuous variables and a Chi-square test with Yate's correction was used to analyze clinical variables and the two tailed Fisher's exact test was used when numbers were small. A *P* value < 0.05 was considered to be statistically significant.

RESULTS

Of 175 patients, 52 patients were aged \geq 60 years (group I, age < 60 years; group II, age \geq 60 years) and 105 were men. Most patients presented with right upper quadrant abdominal pain, jaundice and fever with chills. Of 175 patients, right upper quadrant abdominal pain and fever were more common in group I compared to group II (Table 1). Fever was present in 38 of 52 patients of group II compared to 120 of 123 patients in group I. However, high fever (fever \geq 38.0°C) was more common in patients of group I (118/120 *vs* 18/38) (Table 1). Of 175 patients, hypotension (5/123 *vs* 13/52), altered sensorium (3/123 *vs* 19/52), peritonism (22/123 *vs* 14/52) and renal failure (5/123 *vs* 14/52) were more common in patients of group II compared to patients with group I.

Table 2 Etiology and site of biliary obstruction in common bile duct

Etiology of biliary obstruction	Site of biliary obstruction	Group I <i>n</i> = 123 (%)	Group II <i>n</i> = 52 (%)
Benign causes (<i>n</i> = 138)			
Common bile duct stone	Lower	104 (84.5)	18 (34.6) ^a
Chronic pancreatitis	Lower	2 (1.6)	0
Post operative biliary stricture	Upper	7 (5.7)	0
Hydatid cyst rupture with fistula	Upper	4 (3.2)	0
Rupture liver abscess with fistula	Upper	3 (2.4)	0
Malignant causes (<i>n</i> = 37)			
Gallbladder carcinoma	Upper	2 (1.6)	14 (27) ^a
Cholangiocarcinoma	Upper + middle	0	5 (9.6) ^a
Periampullary carcinoma	Lower	1 (0.8)	15 (28.8) ^a

Table 3 Laboratory parameters before endoscopic biliary drainage (mean ± SD)

	Group I (<i>n</i> = 123)	Group II (<i>n</i> = 52)	<i>P</i>
Total leucocyte count, × 10 ⁹ /L	28.6 ± 6.4	20.4 ± 4.6	< 0.01
Total bilirubin, mg/dL	12.4 ± 8.2	20.6 ± 10.4	< 0.01
Aspartate aminotransferase, IU/L	62 ± 24	58 ± 20.6	NS
Alanine aminotransferase, IU/L	52 ± 18.4	56 ± 14.4	NS
Alkaline phosphatase, IU/L	402 ± 196	1126 ± 644	< 0.01
Serum albumin, g/dL	3.6 ± 0.8	3.8 ± 0.8	NS

Some patients in group II presented with hypotension or altered sensorium despite no or mild abdominal pain and low grade fever.

Etiology and site of biliary obstruction in 175 patients are summarized in Table 2. The biliopancreatic malignancy was a common cause of biliary obstruction in patients of group II (*n* = 34) compared to benign diseases in group I (*n* = 120) (Table 2).

Of 175 patients, 25 patients had other associated comorbid systemic conditions (group I, *n* = 4 and group II, *n* = 21) like diabetes mellitus (*n* = 10), hypertension (*n* = 12), coronary artery disease (*n* = 6), cerebrovascular accidents (*n* = 4), chronic obstructive pulmonary disease (*n* = 4) and pulmonary tuberculosis (*n* = 3) in group II patients compared to pulmonary tuberculosis (*n* = 2), rheumatic heart disease (*n* = 1), diabetes mellitus (*n* = 1), hypertension (*n* = 1) and thyrotoxicosis (*n* = 1). Laboratory investigation revealed higher elevation of leucocytes in group I and higher elevations of bilirubin and alkaline phosphatase levels in group II patients (Table 3). Blood cultures were positive in 70 cases (40%, group I, *n* = 50; group II, *n* = 20) with organisms such as *Escherichia Coli* (*n* = 50; group I, *n* = 38; group II, *n* = 12), *Klebsiella* (*n* = 12; group I, *n* = 9; group II, *n* = 3), *Acinobacter* (*n* = 13; group I, *n* = 9; group II, *n* = 4), *Proteus* (*n* = 9; group I, *n* = 6; group II, *n* = 3) and *Pseudomonas* (*n* = 9; group I, *n* = 6; group II, *n* = 3). Twenty-three patients had mixed infections. The biliary obstruction was most commonly found to be in the lower part of common bile

Table 4 Indications of biliary drainage

Indication	Group I <i>n</i> = 123 (%)	Group II <i>n</i> = 52 (%)	<i>P</i>
Fever ≥ 38.0°C	118 (96)	18 (34.6)	< 0.01
Hypotension	5 (4)	13 (25)	< 0.01
Peritonism	22 (18)	14 (26.9)	< 0.01
Altered sensorium	3 (2.6)	19 (36.5)	< 0.01
Failure to improve with conservative management	2 (1.6)	20 (38.4)	< 0.01

duct in both group I (*n* = 107) and group II (*n* = 33) patients. Indications for biliary drainage were any one of the following either singly or in combination: a fever of ≥ 38.0°C (*n* = 136), hypotension, with systolic blood pressure < 100 mm Hg (*n* = 18), right upper quadrant abdominal pain with guarding (peritonism) (*n* = 36), impaired level of consciousness (*n* = 22), and failure to improve within 72 h of conservative management (*n* = 22). High grade fever was more common indication of biliary drainage in group I and hypotension, altered sensorium, peritonism and failure to improve within 72 h of conservative management were more common indications of biliary drainage in group II patients (Table 4). None of the patients had undergone prior endoscopic or percutaneous transhepatic biliary drainage. After hospital admission, ERCP and endoscopic biliary drainage were performed in patients with severe acute cholangitis after a median interval of 20 h (range 3-42 h) in 153 patients and after a median interval of 96 h (range 72-106 h) in 22 patients who failed to respond to conservative management.

Endoscopic biliary drainage was achieved in 172 patients without any significant age related difference in the success rate. A nasobiliary drain was placed in 80 patients (56 group I, 24 group II) and a stent was placed in 92 patients (64 group I, 28 group II). ERCP with biliary drainage therefore could be achieved in 120 patients in group I and 52 patients in group II. Abdominal pain, fever, jaundice, hypotension, altered sensorium, peritonism and renal failure improved after median time of 5 d in all the 120 patients in group I (range 2-15 d) and after median time of 10 d in 47 patients of group II (range 3-20 d). Similar normalization of leucocyte count was seen after a median time of 7 d (range 3-20 d) in 120 patients in group I and after a median time of 15 d (range 5-26 d) in 47 patients in group II. There were no immediate ERCP related complications in either group of patients. There were no episodes of displacement, kinking or occlusion of NBD or of occlusion or migration of stent.

Five patients (carcinoma gallbladder *n* = 3, CBD stones *n* = 2) died in group II after 3, 5, 6, 9 and 10 d of endoscopic biliary drainage. All the five patients were subjected to endoscopic biliary drainage (2 NBD, 3 stent) after they failed to respond to conservative management for 72 h. All the five patients died because of uncontrolled cholangitis and septicemia despite antibiotics and successful endoscopic biliary drainage.

There was a higher mortality in patients in group II despite successful biliary drainage (0/120 vs 5/52). There was no significant difference in mortality between group

Table 5 Clinical characteristics, indications and results of biliary drainage in patients with acute cholangitis due to stones in common bile duct ($n = 122$)

	Group I $n = 104$ (%)	Group II $n = 18$ (%)	<i>P</i>
Clinical parameters			
Fever	102 (98)	10 (55.5)	< 0.01
Jaundice	101 (97.1)	14 (77.7)	< 0.01
Altered sensorium	1 (0.96)	8 (44.4)	< 0.01
Hypotension	1 (0.96)	5 (27.8)	< 0.01
Peritonism	12 (11.5)	6 (33.3)	< 0.01
Renal failure	3 (2.8)	7 (38.9)	< 0.01
Indications of biliary drainage			
Fever $\geq 38.0^{\circ}\text{C}$	102 (98)	6 (33.3)	< 0.01
Hypotension	1 (0.96)	5 (27.8)	< 0.01
Peritonism	12 (11.5)	6 (33.3)	< 0.01
Altered sensorium	1 (0.96)	8 (44.4)	< 0.01
Failure to improve with conservative treatment	1 (0.96)	7 (38.9)	< 0.01
Results of biliary drainage			
Time taken for improvement			
Median (range) days	5 (2-15)	10 (3-20)	< 0.01
Mortality	0	2	< 0.01
Length of hospital stay			
Mean (range) days	8.2 (7-20)	16.4 (7-30)	< 0.01

II patients with (2/21) and without (3/31) concurrent diseases. Also there was no difference in mortality between group II patients with benign (2/18) and malignant causes (3/34) of biliary obstruction. Length of hospital stay was significantly longer in group II patients (16.4 ± 5.6 , range 7-30 d) than in group I patients (8.2 ± 2.4 , range 7-20 d). Altered sensorium, hypotension, peritonism, mortality and length of hospital stay were more in group II patients with CBD stones ($n = 18$) than in group I patients with CBD stones ($n = 104$) (Table 5).

DISCUSSION

Older age has been considered a risk factor for increased morbidity and mortality rates in the treatment of acute cholangitis^[21,22]. However there is little specific information available on clinical features and treatment of acute cholangitis in the elderly. In present study elderly patients with acute cholangitis were found to have relatively higher incidence of severe cholangitis, hypotension, altered sensorium, peritonism and renal failure. Symptoms in elderly patients did not correlate with the severity of acute cholangitis. Many elderly patients with severe cholangitis presented with deceptively mild symptoms. Presence of abdominal pain and high grade fever was less common in elderly patients compared to young patients. Almost 40% of elderly patients had low grade fever and nearly 1/4 did not have fever. Therefore early diagnosis of acute cholangitis based on symptoms alone was difficult as reported earlier^[21]. However on examination many elderly patients had features suggestive of severe cholangitis in form of jaundice, hypotension, altered sensorium, peritonism and associated renal failure. All the elderly patients showed abnormal results of laboratory tests. However rise in leucocyte count was less compared to leucocytosis in younger patients and bilirubin and alkaline

phosphatase levels were higher in elderly patients than in younger patients. This could be due to biliopancreatic malignancy, a common cause of biliary obstruction in elderly patients compared to benign diseases in younger patients. Elderly patients also had a higher incidence of coexisting medical problems like cardiovascular, pulmonary, neurological and other systemic diseases. Due to the high incidence of severe cholangitis, features like hypotension and altered sensorium and associated other concurrent systemic diseases, the management of acute cholangitis in the elderly becomes difficult^[21].

Urgent endoscopic biliary drainage has been recommended for severe acute cholangitis or cholangitis that does not respond to conservative treatment^[1,3-20]. Endoscopic biliary drainage including endoscopic sphincterotomy, nasobiliary drainage and stenting has been advocated as safe and effective measure for the treatment of acute cholangitis with a mortality ranging from 2% to 8%. In present series, urgent endoscopic biliary drainage yielded favorable outcome for all the young patients. However endoscopic biliary drainage for elderly patients was associated with significant mortality. Earlier studies have shown that the success rates of therapeutic ERCP are similar between elderly and young patients^[24,25]. However the morbidity and mortality associated with therapeutic ERCP are significantly greater in the elderly than in young patients^[23-25]. There were no serious complications of endoscopic biliary drainage procedures in both elderly and younger patients. In elderly patients higher morbidity and mortality even after biliary drainage are considered due to advanced acute cholangitis, exacerbation of concurrent medical illnesses, unsuccessful biliary drainage procedures, underlying biliopancreatic malignancy as a cause of biliary obstruction, complications characteristic of elderly such as delirium, pneumonia or atelectasis and need for prolonged ventilatory support after the endoscopic drainage procedures^[21-23]. Elderly patients are often unable to tolerate endoscopic procedures because they are critically ill or uncooperative. In this situation, the endoscopic procedures can be performed safely and successfully under general anesthesia and endotracheal intubation^[21,25]. However none of the elderly patients in present series required general anesthesia, endotracheal intubation and ventilatory support after endoscopic procedures for biliary drainage.

In present study, the clinical profile and response to endoscopic biliary drainage in elderly patients was similar in patients having biliary obstruction due to benign and malignant causes. Elderly patients with CBD stones had different clinical profile, poorer response to biliary drainage and higher mortality as compared to young patients with CBD stones. In present series, all the five elderly patients were subjected to endoscopic biliary drainage after they failed to respond to conservative management for 72 h. All the five patients died because of uncontrolled cholangitis and septicemia despite antibiotics and successful endoscopic biliary drainage. None of the patients who underwent immediate endoscopic biliary drainage because of high grade fever or associated features like hypotension, altered sensorium and peritonism had died. It is possible that patients with low grade fever and

without features like hypotension, altered sensorium and peritonism also had severe acute cholangitis which was not clinically evident. Therefore they did not fit into indications of immediate endoscopic biliary drainage without waiting for assessment of response to conservative management for 72 h and accounted for 25% (5/20) of mortality in subgroup of elderly patients not responding to conservative treatment for 72 h. Some of the elderly patients presented with hypotension or altered sensorium despite absent or mild abdominal pain and without or with low grade fever suggesting thereby that elderly patients may have severe acute cholangitis without fever or with low grade fever and should be subjected to immediate endoscopic biliary drainage without waiting for 72 h to assess the response to conservative treatment for 72 h. Higher mortality has earlier been reported in patients with acute cholangitis who fail to respond to antibiotics and therapeutic intervention is performed after 72 h of hospital admission^[23,26]. It has been found that many times it is not always clinically apparent which patients will respond to medical treatment alone and which will require urgent biliary drainage. The mortality rate is high for patients who undergo delayed biliary drainage after failure of medical therapy^[23,26]. In elderly patients, the levels of bilirubin and alkaline phosphatase were significantly higher than in young patients. It has been reported that higher levels of bilirubin and alkaline phosphatase are associated with higher mortality in patients with acute cholangitis^[22]. The differences in clinical profile, laboratory parameters and response to biliary drainage in elderly patients can not be attributed to differences in type of bacteria causing cholangitis because the bacteriological spectrum was similar in elderly and young patients in present study.

Elderly patients with acute cholangitis have a high incidence of severe cholangitis, concomitant medical illnesses, hypotension, altered sensorium, peritonism, renal failure and higher mortality even after successful biliary drainage.

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CASE REPORT

Portal thrombosis and steatosis after preoperative chemotherapy with FOLFIRI-bevacizumab for colorectal liver metastases

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INTRODUCTION

Surgical resection of colorectal liver metastases is nowadays a standard of care for resectable disease with 5-year survival rate approaching 60%^[1-3]. Because of several theoretical benefits, preoperative systemic chemotherapy has been frequently used to downsize the disease.

We report here the development of two complications, partial portal vein thrombosis and hepatic steatosis with lobular inflammation, during the course of preoperative chemotherapy with FOLFIRI plus bevacizumab for colorectal liver metastases, and discuss the surgical management and implications.

Abstract

In order to discuss the role of preoperative chemotherapy for colorectal liver metastases, which is used frequently before hepatic resection, even in patients with resectable disease at presentation, we herein report the development of two complications, partial portal vein thrombosis and hepatic steatosis with lobular inflammation, during the course of preoperative chemotherapy with FOLFIRI plus bevacizumab for colorectal liver metastases, which recognition led to timely discontinuation of chemotherapy as well as a change in the surgical strategy to resect the tumors and the damaged liver through advanced techniques. We conclude that duration of treatment and drug doses and combinations may impact the development of chemotherapy-induced liver injury. Surgeons and medical oncologists must work together to devise safe, rational, and oncologically appropriate treatments for patients with multiple colorectal liver metastases, and to improve the understanding of the pathogenesis of chemotherapy-induced liver injury.

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Key words: Chemotherapy; Colorectal liver metastases; Resection

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CASE REPORT

A 61-year-old woman was referred for treatment of synchronous multiple bilobar colorectal liver metastases. Six months after a laparoscopic right hemicolectomy for a pT3N2 colon cancer, a multiphase spiral computed tomography (CT) performed without and with intravenous (IV) contrast revealed bilateral disease with a total of five lesions distributed in segments I, II, IVA, VI and VII amenable to hepatic resection (Figure 1). Because of the known high risk of disease recurrence after liver resection for multiple bilobar and synchronous metastases in patients with N2 classification of primary tumor, the patient was treated with six courses of preoperative chemotherapy with FOLFIRI (irinotecan, 5-fluorouracil, and high-dose leucovorin) plus bevacizumab.

Repeat CT performed without and with IV contrast 3 wk after the completion of chemotherapy with bevacizumab showed downsizing of the lesions in segments I, IVA, VI-VII, and stable disease in the segment II. Non-contrast images also showed the development of diffuse fatty infiltration of the liver with sparing of segments V and VIII while the images acquired after IV contrast administration revealed absent portal perfusion of the right anterior sector associated with right anterior portal branch occlusion. The lack of portal flow to segments V and VIII explained sparing from steatosis and increased arterial flow to these segments. Some compensatory hypertrophy of the left liver was evident (Figure 2). The patient had no evidence of hypercoagulation and no other known risk factors for thrombosis. Even though this patient experienced downsizing of bilobar liver metastases, the new finding



Figure 1 Contrast enhanced CT at the level of the portal bifurcation before treatment shows metastasis in segment I (arrowhead), opacified right anterior and posterior segmental portal veins (white arrows), and opacified middle and right hepatic veins (open arrows).

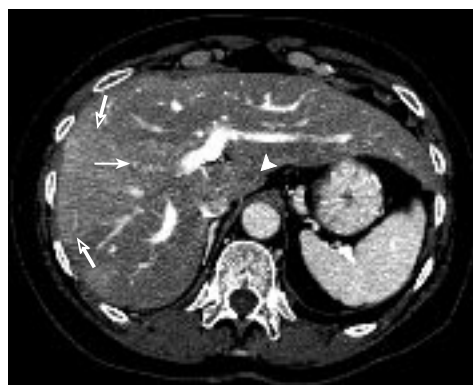


Figure 2 Contrast enhanced CT at the level of the portal bifurcation after treatment shows improved hepatic metastasis in segment I (arrowhead), non-visualization of the thrombosed anterior right segmental portal vein (arrow), opacified posterior segmental portal veins, middle and right hepatic veins, and wedge shaped increased enhancement (open arrows) of the anterior sector secondary to portal vein occlusion.

of portal vein thrombosis led to discontinuation of chemotherapy and reassessment of the treatment plan. Prior to occurrence of portal vein thrombosis, a partial right hepatectomy plus resection of the metastases in segments IVA, II and I, was planned. The finding of right portal vein thrombosis led to a change in operative plan to include resection of the segments involved both by tumor and by portal vein thrombosis in a 2-stage fashion. At the first stage, segmental resection of the lesions in segments I and II was performed, preserving the majority of the parenchyma of the lateral bisegment. Three-dimensional liver volumetry revealed the planned remnant liver (bisegment II/III) was of inadequate volume to permit resection of the right liver plus segment IV. Therefore, in order to increase volume and function of the liver remnant prior to the 2-stage resection, percutaneous right portal vein embolization extended to segment IV was performed. Repeat CT volumetry after 4 wk revealed adequate liver remnant hypertrophy, and the remaining diseased liver was resected by extended right hepatectomy (resection of Couinaud segments IV through VIII). The postoperative course after each stage was uneventful. The pathological review of the specimen revealed 50% necrosis in the tumors and moderate-severe steatosis with lobular inflammation in the resected liver.

DISCUSSION

We report the development of partial portal vein thrombosis in combination with hepatic steatosis and lobular inflammation (steatohepatitis) during the course of preoperative chemotherapy with FOLFIRI plus bevacizumab for colorectal liver metastases. This thrombotic complication, which occurred during chemotherapy, was possibly not because of the presence or treatment effect of a metastatic lesion since no tumor was associated with the thrombosed portal vessel. Absence of previous data on vascular events in association with 5-fluorouracil alone, a drug which has been in use for more than 50 years, raises the possibility of an event associated with bevacizumab and/or irinotecan treatment.

Bevacizumab is a humanized recombinant murine monoclonal antibody to vascular endothelial growth

factor A (VEGF-A) that competitively blocks binding of VEGF-A to its receptors, resulting in inhibition of angiogenesis. Bevacizumab has been reported to be associated with delayed wound healing, venous and arterial thromboembolism; however, to our knowledge bevacizumab has not been reported to cause portal venous thrombosis^[4-8]. The proposed mechanism of bevacizumab-related thrombosis is complex-both hemorrhagic and thrombotic events may be involved. Briefly, bevacizumab by antagonizing VEGF's functions might decrease the renewal capacity of endothelial cells in response to trauma, leading simultaneously to a tendency to bleeding and thrombosis^[9,10].

Preliminary evidence that irinotecan may be associated with arterial and venous thrombotic events has been reported as well, but portal vein thrombosis has not been reported previously and the pathophysiology of irinotecan-related thrombotic events remains unclear^[6,11].

The baseline incidence of thrombosis in cancer patients is significant, and the development of portal vein thrombosis in our patient cannot be definitively attributed to chemotherapy^[12]. However, the pattern of thrombosis, the occurrence during chemotherapy treatment, and the absence of any vascular complications after colectomy, portal vein embolization or 2 sequential hepatectomy procedures, suggest that combination of FOLFIRI and bevacizumab contributed to this complication.

Histopathologic review of the resected liver confirmed not only moderate-severe steatosis consistent with the CT findings, but also revealed lobular inflammation^[13]. Steatosis plus lobular inflammation can progress to steatohepatitis, which has been associated with preoperative chemotherapy with irinotecan, and may raise the risk of death after liver surgery^[14-17]. Adverse outcomes related to chemotherapy-induced injury likely relate to a decreased regenerative capacity of injured hepatocytes in response to major hepatectomy, possibly through alterations of nuclear factors such as nuclear factor-kappa B, which is crucial for the priming phase of liver regeneration^[18].

In the patient reported, who had resectable disease at presentation, development of both portal vein thrombosis

and hepatic steatosis suggests that chemotherapy treatment contributed to these events and raised the possibility that curative surgery would not be possible. Fortunately, the recognition of these complications led to timely discontinuation of chemotherapy as well as a change in the surgical strategy to resect the tumors and the damaged liver through advanced techniques (portal vein embolization, extended hepatectomy, 2-stage approach) in an effort to minimize morbidity.

Chemotherapy will be used with more frequency before hepatic resection. Duration of treatment and drug doses and combinations may impact the development of chemotherapy-induced liver injury. Surgeons and medical oncologists must work together to devise safe, rational, and oncologically appropriate treatments for patients with multiple colorectal liver metastases, and to improve the understanding of the pathogenesis of chemotherapy-induced liver injury.

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Gallbladder tuberculosis: False-positive PET diagnosis of gallbladder cancer

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Abstract

Gallbladder tuberculosis (GT) is an extremely rare disease, and very few cases have been reported in the literature. The first case of GT was described in 1870 by Gaucher. A correct preoperative diagnosis of GT is unusual, and it is frequently confused with various gallbladder diseases. We present a new case of a patient who underwent surgery with the preoperative diagnosis of gallbladder cancer after a false positive positron emission tomography scan in the diagnostic work-up.

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Key words: Positron emission tomography; Gallbladder; Tuberculosis; Cancer; Review

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INTRODUCTION

Abdominal tuberculosis is common in developing countries but gallbladder tuberculosis (GT) is an extremely rare disease^[1,2], and very few cases have been reported in the literature^[1]. The first case of GT was described in 1870 by Gaucher.

A correct preoperative diagnosis of GT is unusual, and it is frequently confused with various gallbladder diseases. We present a new case of a patient who underwent surgery with the preoperative diagnosis of gallbladder cancer after a false positive positron emission tomography (PET) scan in the diagnostic work-up.

CASE REPORT

A 64-year-old man was admitted to our department for abdominal pain and jaundice. Abdominal examination revealed a palpable mass in right hypochondrium. Laboratory tests were normal. A gallbladder mass was observed on ultrasound. A computed tomography (CT) scan showed a thickened gallbladder wall with possible infiltration of liver parenchyma and duodenum, and we proposed a diagnosis of advanced neoplasm of gallbladder (Figure 1). A PET scan showed a hypermetabolic area in the right liver lobe in the usual location of the gallbladder, infiltrating the liver parenchyma (Figure 2) and reaffirming our suspected diagnosis of advanced neoplasm of gallbladder.

We performed a subcostal laparotomy and observed a very large tumor that involved gallbladder and duodenum. A biopsy of the gallbladder wall was taken, and the histologic perioperative study reported gallbladder tuberculosis with no signs of malignancy. We then performed a cholecystectomy and observed the presence of cholecistoduodenal fistulae, so that we closed the duodenum. An exhaustive examination of the abdominal cavity revealed no other foci of tubercular infection. The definitive histologic study showed granulomatous reaction with the presence of multinucleated giant cells and caseum necrosis compatible with GT.

The postoperative course was uneventful. A Mantoux test was positive. Other techniques (chest X-ray, bronchoscopy) showed no tubercular infection in other organs. Antitubercular drugs were prescribed for six months.

DISCUSSION

The gallbladder is highly resistant to tubercular infection, and the presence of cholelithiasis and cystic duct obstruction is essential for the development of GT^[1,2]. About 70% of GT cases are accompanied by gallstones^[2]. Four types of GT have been described, according to whether only the gallbladder is involved or there is generalized tuberculosis and whether the gallbladder mucosa is histologically involved^[1]. GT often occurs together with other intra-abdominal tuberculosis, usually in women over 30 years of age^[2]. However, in the present case it was restricted to the gallbladder, because the duodenum was only locally involved. The route of infection may be peritoneal, hematogenous or lymphatic^[2]. Histologically, the lesion may be in the form of a localized



Figure 1 CT scan suggestive of image of gallbladder neoplasm.

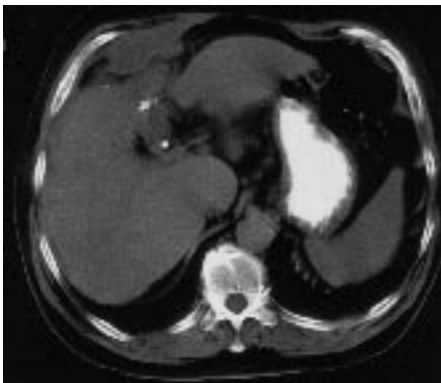


Figure 2 High uptake in gallbladder fossa (false positive for gallbladder cancer).

ulceration or there may be typical tuberculosis nodules in the wall of varied size and numbers.

A wide spectrum of symptoms have been described in patients with GT, including abdominal pain in right hypochondrium, weight loss, fever, anorexia, diarrhea, nausea, vomiting and a palpable abdominal mass^[2]. Anemia, elevated ESR and positive tuberculin test are usually found in laboratory examinations.

The correct diagnosis of GT is difficult, and it is usually made after a cholecystectomy^[1]. Ultrasound and CT may show an enlarged gallbladder, a thickened gallbladder wall, soft tissue masses, or nodular lesions, although neither ultrasound nor CT lesions are specific^[2]. The diagnosis of tuberculosis in other abdominal organs (liver, spleen, lymph nodes or peritoneum) is very helpful for diagnosing GT.

The differential diagnosis of GT includes acute and chronic cholecystitis, polypoid lesions and gallbladder carcinoma^[2]. The presence of a mass that fills the gallbladder associated with cholelithiasis is indistinguishable from carcinoma of the gallbladder^[1]. Moreover, both GT and carcinoma can give rise to regional lymph nodes. The presence of liver metastasis or liver infiltration suggests the presence of a gallbladder carcinoma. On the other hand, lung lesions or mesenteric thickening is frequent in patients with tubercular infection.

PET is a very accurate diagnostic method for cancer.

PET can image the metabolic differences between normal and malignant cells using tumor-seeking tracers^[3]. According to some authors, it is difficult to distinguish patients with lung cancer from those with pulmonary tuberculosis due to the high uptake rate of patients with active tuberculosis^[3,4]. This is because an increased glycolysis of macrophages, cells present in tubercular infection, results in a high FDG uptake value^[3]. 11C-choline tracer is more accurate than 18F-fluorodeoxyglucose for making the differential diagnosis between tubercular infection and cancer^[3].

There has been little experience with PET as a diagnostic tool in gallbladder cancer but the first published reports are fairly encouraging, showing a sensitivity of 75%^[5,6]. At our department, a prospective trial is in progress to establish the sensitivity of PET scan in patients with suspicion of gallbladder cancer based on CT scan. The present patient was included in this trial and the results obtained appeared to confirm the suspected diagnosis of gallbladder cancer. To our knowledge, this is the first case of a false positive PET scan in a gallbladder cancer due to GT. There is one previous report of pancreatic tuberculosis that was misdiagnosed as pancreatic cancer after a positive PET scan^[7].

Tuberculosis was common in Spain in the past but is now an uncommon finding in immunocompetent patients, and the GT diagnosis was therefore unexpected. In the countries with a high rate of tuberculosis infection, the possibility of a tuberculosis infection should be considered in patients suspected of a hepatobiliary cancer (liver, pancreas or gallbladder) with a positive PET if the patient has a positive Mantoux test or a history of tuberculosis.

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Isolated splenic vein thrombosis secondary to splenic metastasis: A case report

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INTRODUCTION

Isolated splenic vein thrombosis is a rare clinical syndrome that may lead to left-sided portal hypertension. Metastasis to the spleen from solid tumors is also considered rare. When identified it usually occurs in the setting of widely disseminated diseases. We report a case of isolated splenic vein thrombosis secondary to splenic metastasis of an adenocarcinoma of the colon. The splenic lesion was detected before the resection of the primary colon lesion during a complete metastatic work-up.

CASE REPORT

A 49-year-old woman presented complaining of abdominal pain in May 2003. There was no relevant medical history. Her performance status was not impaired. Physical examination revealed upper abdominal tenderness with mild guarding. Laboratory findings revealed anemia with hemoglobin of 84 g/L and hematocrit of 29.9%. Esophagogastroduodenoscopy revealed no remarkable finding. Colonoscopy revealed ascending colon cancer of 7 cm in diameter. The level of tumor markers was elevated with carcinoembryonic antigen of 36.7 µg/L and CA19-9 of 1870 kU/L. Computed tomography of the abdomen showed splenic metastasis (Figure 1A), but no metastasis in the liver. Contrast-enhanced computed tomography and angiography showed occlusion of the splenic vein, a shadow defect at the portal vein, and development of collaterals, which suggested tumor thrombosis extending from the splenic vein to the portal vein (Figures 1B, 2A, B).

We diagnosed her as having an ascending colon cancer with splenic metastasis and splenic vein tumor thrombosis (T3NXM1, Stage IV). She underwent right hemicolectomy, splenectomy, and distal pancreatectomy. The tumor of the spleen was adherent to the left hemidiaphragm, so a small segment of the diaphragm was removed en bloc. The portal vein was resected at its junction with the splenic vein and reanastomosed.

Histological findings showed a moderately differentiated adenocarcinoma of the ascending colon (Figure 3A) that invaded into serosa, with one of the five lymph nodes being positive for metastatic adenocarcinoma.

Abstract

A 49-year-old, previously healthy woman sought treatment for abdominal pain. Colonoscopy revealed ascending colon cancer. Computed tomography and angiography showed splenic metastasis and thrombosis extending from the splenic vein to the portal vein. She underwent right hemicolectomy, splenectomy, and distal pancreatectomy. Histological findings showed no malignant cell in the splenic vein which was filled with organizing thrombus. We postulate the mechanism of splenic vein thrombosis in our case to be secondary to the extrinsic compression of the splenic vein by the splenic metastasis or by the inflammatory process produced by the splenic metastasis. In conclusion, we suggest that splenic metastasis should be added to the list of differential diagnosis which causes splenic vein thrombosis. In the absence of other sites of neoplastic disease, splenectomy seems to be the preferred therapy because it can be performed with low morbidity and harbors the potential for long-term survival.

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Key words: Colon cancer; Splenic metastasis; Splenic vein occlusion; Splenic vein thrombosis

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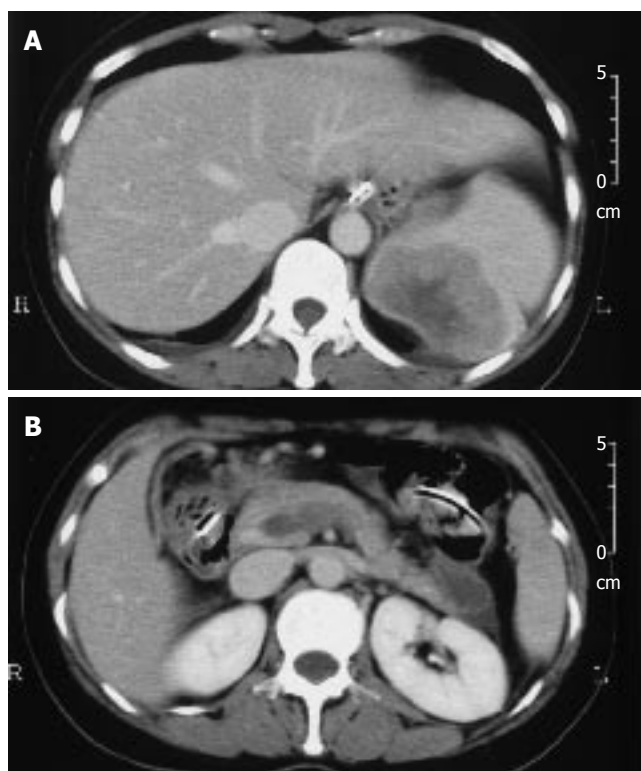


Figure 1 Contrast-enhanced computed tomography of the abdomen. **A:** Enlarged spleen with hypodense lesion in the superior spleen which was considered to be splenic metastasis; **B:** Hypoattenuating thrombus in the splenic vein and the portal vein.

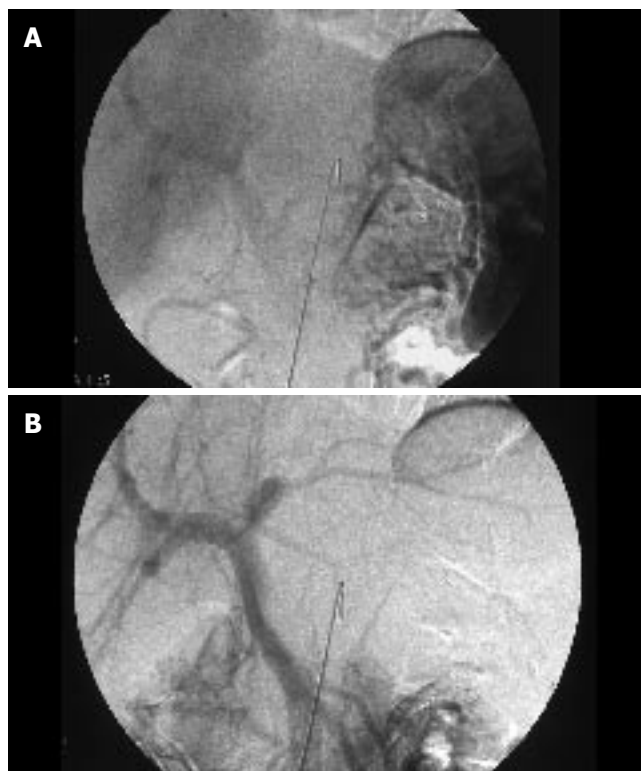


Figure 2 Digital subtraction angiography. **A:** The venous phase of the celiac artery angiogram reveals occlusion of the splenic vein and development of collaterals; **B:** The venous phase of the superior mesenteric artery angiogram reveals a defect shadow in the portal vein at its junction with the splenic vein.

The tumor of the spleen consisted of a moderately

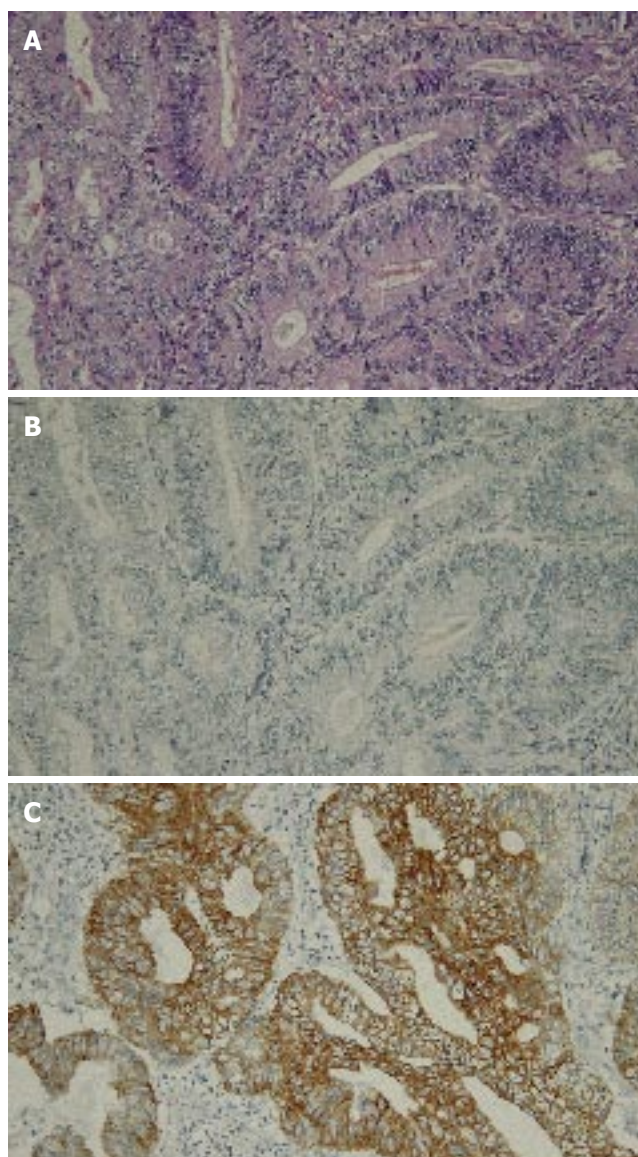


Figure 3 Histological findings of the tumor of the ascending colon. **A:** Moderately differentiated adenocarcinoma (HE, x 20); **B:** Immunostaining for cytokeratin 7 showing a negative reaction (x 20); **C:** Immunostaining for cytokeratin 20 showing a positive reaction (x 20).

differentiated adenocarcinoma similar in histology to the tumor of the ascending colon (Figure 4A). These sections were stained with immunohistochemical reactions of antibodies to cytokeratins 7 and 20 (Figures 3B, C, 4B, C). Negative staining for cytokeratin 7 and positive staining for cytokeratin 20 was consistent with splenic metastasis of an adenocarcinoma of the colon^[1].

On the contrary to the preoperative diagnosis, no malignant cell was recognized in the splenic vein which was filled with organizing thrombus (Figure 5).

The postoperative course was uneventful. Two years and three months later, the patient is alive and well except for a nodule in the Douglas fossa which seems to be metastasis.

DISCUSSION

Isolated splenic vein thrombosis is a rare clinical syndrome that causes left-sided portal hypertension. It may lead to

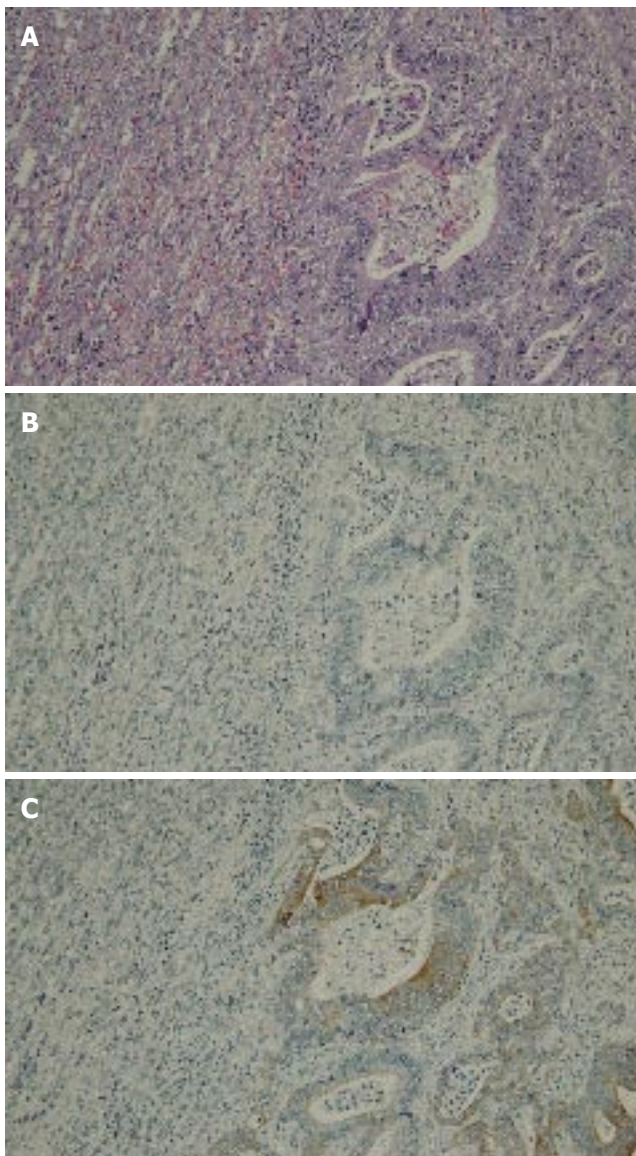


Figure 4 Histological findings of the tumor of the spleen. **A:** Moderately differentiated adenocarcinoma (HE x 20); **B:** Immunostaining for cytokeratin 7 showing a negative reaction (x 20); **C:** Immunostaining for cytokeratin 20 showing a positive reaction (x 20).

bleeding from gastric varices. The majority of splenic vein thrombosis are the result of pancreatic pathologies, including acute and chronic pancreatitis, pancreatic pseudocyst, pancreatic tumor and abscesses^[2]. Other reported causes are traumas, umbilical vein catheterizations, lymphomas and sarcomas, retroperitoneal fibrosis, gastric surgeries, splenic artery aneurysms, myeloproliferative diseases, hereditary thrombocythemia^[3], renal diseases including renal cell carcinomas^[4] and benign renal cysts^[5], and tuberculous lymphadenitis^[6]. Our case did not have these diseases, and so we postulate the mechanism of splenic vein thrombosis in our case to be secondary to the extrinsic compression of the splenic vein by the splenic metastasis or by the inflammatory process produced by the splenic metastasis. To our knowledge, there has been no report of isolated splenic vein thrombosis secondary to

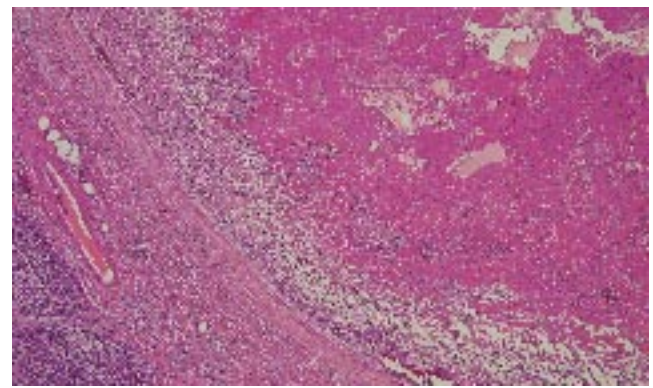


Figure 5 Histological findings of the splenic vein filled with not tumor thrombus, but organizing thrombus. The elastic tissue ring is intact (HE, x 4).

splenic metastasis.

Carcinoma of the colon frequently metastasizes to the liver but only occasionally involves the spleen. The spleen is considered an unfavourable site for the development of metastases but the reason for this is not fully understood. Metastatic carcinoma that involves the spleen is usually a manifestation of widely disseminated disease involving multiple organs. Berge conducted an autopsy study on 7246 patients with various types of malignant tumors^[7]. Splenic metastases were found in 21 out of the 1019 (2%) patients with colorectal carcinoma, but all were associated with involvement of other organs as well.

There are no long-term follow-up data of splenectomy for metastasis from colon cancer. Our patient underwent splenectomy, as it would deem to provide the only chance for long-term survival.

In conclusion, we suggest that metastatic tumor of the spleen should be added to the list of differential diagnosis which causes splenic vein thrombosis. Splenic metastasis from adenocarcinoma of the colon is a rare finding. In the absence of other sites of neoplastic disease, splenectomy seems to be the preferred therapy because it can be performed with low morbidity and harbors the potential for long-term survival.

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CASE REPORT

Budd-Chiari like syndrome in decompensated alcoholic steatohepatitis and liver cirrhosis

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Abstract

A rare case of pseudo-Budd-Chiari Syndrome in a patient with decompensated alcoholic liver disease is reported. Although clinical and radiological findings suggested Budd-Chiari Syndrome, the liver biopsy revealed micronodular cirrhosis and absence of histological signs of hepatic outflow obstruction.

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Key words: Pseudo Budd-Chiari; Budd-Chiari syndrome; Liver cirrhosis; Alcoholic steatohepatitis

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INTRODUCTION

Budd-Chiari Syndrome (BCS) is a rare, heterogeneous and potentially lethal condition caused by obstruction of the hepatic venous outflow tract^[1], situated anywhere between the small hepatic venules until the right atrium^[2]. In Western countries, thrombosis from multiples causes is the predominant factor in the etiology of BCS^[3]. The correct diagnosis is very important for the therapeutic approach. Doppler ultrasound, computed tomography (CT) scan, and magnetic resonance imaging (MRI) provide evidence of hepatic vein (HV) thrombosis, with different specificity

and sensitivity^[4-7].

Pseudo-BCS is a condition which until now has not been well known. Only four reports were found in the literature and all of these concerned patients with a mechanical compression of the hepatic outflow due to an enlarged liver and liver cirrhosis.

The diagnosis of pseudo-BCS is difficult, because clinical and radiological findings are extremely similar to BCS, making invasive methods necessary.

We report a case of presumed BCS in a patient with alcoholic liver disease and emphasize the importance of the differential diagnosis for the correct management of these patients.

CASE REPORT

A 49-year-old female was admitted to our hospital with a 6-mo history of abdominal pain, progressive ascites, weight loss, anorexia and fatigue. There was no previous history of GI bleeding, surgery, hemotransfusions or spontaneous abortion. Also, she denied the use of oral contraceptives. Right up until admission day, she had consumed excessive amounts of alcohol for over 8 years. On physical examination we found mild jaundice, anemia, spider nevi, palmar erythema, hepatomegaly of 16 cm below the lower edge of the ribs, moderate ascites and edema. A systolic ejection murmur at the abdominal right upper quadrant was also found. Cardiac and pulmonary auscultation was normal. There was no jugular venous distention or pulsatile liver. Laboratory results are listed in Table 1.

Spontaneous bacterial peritonitis was diagnosed and treated. During endoscopy, three small esophageal varices were found. Doppler ultrasound revealed gross hepatomegaly 20 cm, with only a narrowed middle HV visible and a tributary branch. Absence of flow in the HV was reported. The CT-scan showed the massive hepatomegaly with irregular distribution of contrast, enlarged caudate lobe, and contrast hypercapitation at the left hepatic lobe.

The possibility of hepatocarcinoma plus BCS was considered. Serum viral markers were all negative and α -fetoprotein level was normal (4.61 U/mL).

MRI examination suggested the diagnosis of BCS due to patchy enhancement, absence of left and right HV and a narrowed middle HV with two narrowed tributary branches. An arterio-portal shunt was seen and hepatocarcinoma was excluded (Figure 1).

The diagnosis of BCS was made with a high degree of certainty by radiological exams. The search for hypercoagulable states was all negative.

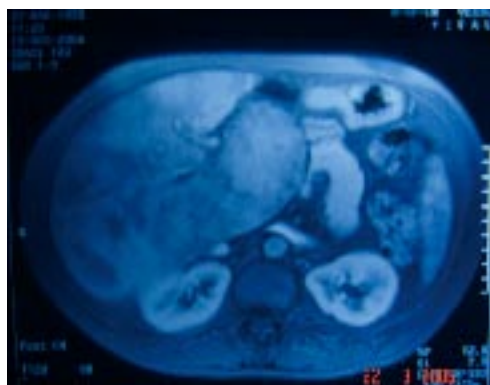


Figure 1 MRI in arterial phase showing a massive hepatomegaly with enlarged caudate lobe and patchy enhancement of contrast. A branch of portal vein is shown (arterio-portal shunt).

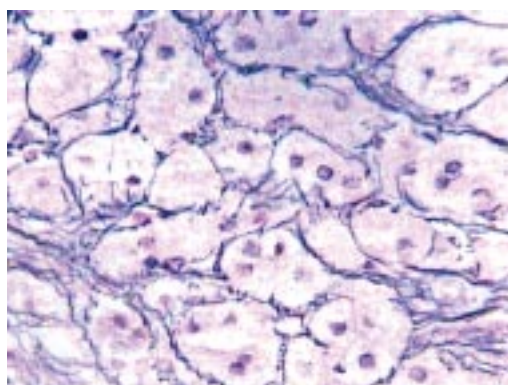


Figure 2 Liver biopsy reveals perisinusoidal and pericellular fibrosis (Gomori's reticulin stain, original magnification x 100).

Due to the patient's previous history and clinical condition, a liver biopsy was performed in order to clarify the extent of liver damage due to alcohol abuse as well as venous occlusion.

The liver biopsy showed micronodular cirrhosis, perisinusoidal and pericellular fibrosis (Figure 2). There was neither pericentral congestion nor other histological signs of hepatic outflow congestion and the use of anticoagulant therapy was not indicated. The venography was not performed due to the results of the liver biopsy.

The patient was discharged four weeks after admission with improvement of her clinical condition and laboratory results (Table 1).

DISCUSSION

BCS is a rare disease, varied in cause, presentation and progression, with different diagnostic and therapeutic approaches. The radiological methods are part of the diagnostic work-up for BCS^[1,8].

The first-line test is Doppler ultrasound of the liver^[1] that has a sensitivity of 87.5% in the diagnosis of BCS^[4]. The presence of a caudate vein equal or larger than 3 mm in diameter^[9] or abnormalities in the flow of the HV outflow tract are suggestive of BCS^[5,10].

MRI is the second-line test for BCS^[1]. It permits differentiation between all forms of BCS^[11]. Due to the high cost and low availability of this test, MRI is only

Table 1 Laboratory results

	Admission day	Discharge day	Normal values
Hemoglobin (mg/dL)	9.7	11.0	12.0-16.0
Mean corpuscular volume (fL)	108.1	103	80.0-96.0
Leukocytes (/mm ³)	21800	10100	4000-11000
Platelets (mil/mm ³)	306	250	140-360
Prothrombin time (s)	17.6	15.2	13
INR	1.52	1.2	1.0-1.4
Aspartate aminotransferase (U/L)	135	44	15-37
Alanin aminotransferase (U/L)	32.7	40	30-65
Gamma-GT (U/L)	907	312	5-85
Bilirubin (mg/dL)	2.73	1.4	0-1.0
Albumin (g/L)	24	36	34-50
Creatinine (mg/dL)	0.5	0.6	0.6-1.3
Alkaline fosfatase (U/L)	217	118	50-136

recommended when the diagnosis is not clear after Doppler ultrasound^[1,8,12].

A CT-scan may show an enlargement of the liver (caudate lobe), with patchy enhancement after contrast; but with indeterminate results due to false-positive in 50% of the cases^[5,7]. Combination of the three techniques in the appropriate clinical setting increases sensitivity to 80%-90%^[13].

In accordance with international guidelines, in BCS, liver biopsy and venography of hepatic veins are indicated when the diagnosis remains uncertain^[1,14]. The third-line investigation is liver biopsy, which provides information for important differential diagnoses^[14]. In current practice venography is rarely considered necessary for establishing a diagnosis and is carried out only when treatment is being planned^[1].

Clinical exams and radiological testing strongly indicated that our patient was suffering from BCS. Due to the radiological results during the clinical evolution, it was no necessary to perform the venography. However, when the possible causes of BCS in our patient were extensively sought, by other means, all of them turned out to be negative.

The hypothesis of hepatocarcinoma was made due to the findings in the CT-scan. But this, too, was excluded after the MRI showed an arterio-portal shunt. It is well known that arterio-portal shunts can be present in a cirrhotic liver and confuse the diagnosis with hepatocarcinoma^[15], as was the case in our patient.

Hypercoagulable states and thrombosis of other causes represent more than 75% of the etiology in patients with BCS^[8]. Other authors suggest that more than 85% of idiopathic causes of BCS are due to myeloproliferative disorders^[16,17].

One doubt in this case was the possibility that alcoholic intake could have been the cause of this BCS or in some way be associated with it. Two reports in the literature show this rare association^[13,18].

Shankel *et al* in 1987 described a patient with BCS and alcoholic liver disease. The patient presented both inferior vena cava (IVC) and nephrotic syndromes. In spite of the BCS diagnosis, absence of thrombus and only a mechanical compression of the IVC was found at

autopsy^[18].

The second report was made by Janssen *et al*, fifteen years later. It described three patients with alcoholic liver disease and BCS, without any thrombotic occlusion. These cases were denominated pseudo-BCS^[13].

Another two cases of pseudo-BCS were reported in association with liver cirrhosis of unknown causes, without HV occlusion findings^[19,20].

Dhawan *et al* in 1978, described a patient with clinical and radiological findings of BCS and concomitant liver cirrhosis. A complete constriction of the right HV and a narrowed left HV were shown to be present due to the hypertrophy of the left lobe of the liver and a regenerative nodule. No thrombotic occlusions were seen at the autopsy^[19]. This was the first paper to introduce the term pseudo-BCS.

Rector *et al* two years later, also reported a patient with liver cirrhosis, probably by non-alcoholic steatohepatitis and BCS. A distortion of the IVC caused by cirrhosis and increased abdominal pressure suggested BCS due to membranous obstruction, but no thrombus or membranous occlusion was found^[20].

All the cases reported had similar clinical, laboratory and radiological features and diagnostics.

Some of the described patients had a good evolution after alcohol withdrawal^[13,18], including our patient. The other patients had fatal outcomes because of severe hepatic damage due to excessive alcohol intake or advanced liver disease^[18,19]. One patient died after unnecessary anticoagulant therapy^[13], which worsened the coagulation profile. No patients had pericentral congestion nor histological signs of hepatic outflow obstruction characteristic of BCS. A mechanical compression of the hepatic outflow due to an enlarged liver was seen in all cases. Histological changes in the architecture of the small hepatic veins were also observed in our patient.

Structural lesions of the hepatic venules, such as veno-occlusive changes, perivenular fibrosis and lymphocytic phlebitis have been well documented in patients with alcoholic liver disease^[21].

Probably the structural lesions at the hepatic venules in association with mechanical compression due to anatomic abnormalities caused by an enlarged liver contributed to the misleading picture of BCS in these patients.

In conclusion the alcoholic steatohepatitis with liver cirrhosis can show a BC-like syndrome. Patients with this rare association denominated pseudo-BCS should be extensively investigated because management will depend on the correct diagnosis. The treatment of pseudo-BCS caused by alcoholic liver disease consists basically in discontinuing alcohol ingestion and generally is associated with a good prognosis.

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A case of hepatocellular carcinoma arising within large focal nodular hyperplasia with review of the literature

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Abstract

Focal nodular hyperplasia (FNH) is a relatively rare benign hepatic tumor, usually presenting as a solitary lesion; however, multiple localizations have also been described. The association of FNH with other hepatic lesions, such as adenomas and haemangiomas has been reported by various authors. We herein report a case of a hepatocellular carcinoma arising within a large focal nodular hyperplasia, in a young female patient.

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Key words: Focal nodular hyperplasia; Hepatocellular carcinoma; Liver tumors

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INTRODUCTION

Focal nodular hyperplasia (FNH) is a relatively rare benign liver tumor, often asymptomatic and discovered incidentally^[1,2]. It occurs in both men and women, but shows a predilection for young women. FNH presents as a solitary lesion in 70% of the cases, while in 30% of patients two to five lesions are present^[3]. Multiple lesions occur rarely^[4-7].

Although an association with the use of oral contraceptives has been shown^[6,7], its pathogenesis is still unclear.

The potential for malignant transformation of FNH into hepatocellular carcinoma (HCC) has not been demonstrated^[8]. However, cases of anatomical adjacency of fibrolamellar carcinoma (FL-HCC) and FNH in the same patient have been described, and some authors have suggested a direct link between the two tumors^[9,10].

The simultaneous presence of HCC in its typical form and FNH is exceptional. To the best of our knowledge, only few cases regarding simultaneous occurrence of FNH and HCC of the liver have been reported in literature^[8,11]. In this case report we describe a case of co-existent FNH and HCC and discuss the clinical management and therapeutic implications.

CASE REPORT

A 23-year-old woman was referred to the Outpatient Department of our hospital with a present history of fatigue and a past medical history of atresia of oesophagus which was reconstructed during her neonatal age and underwent oesophageal dilatations in her adolescence. She has never used oral contraceptives and had no history of hepatitis or alcohol abuse. Physical examination did not reveal any abnormal findings. Laboratory findings including serum α -fetoprotein and carcinoembryogenic antigen levels were within normal ranges. Contrast-enhanced abdominal CT scan showed five lesions in the liver (both left and right lobes), with the largest of the lesions located in the left lobe, an angiomyolipoma in the right kidney and multiple small angiomyolipomas of the left kidney. All liver lesions enhanced greatly during the early arterial phase (Figure 1A and B).

Three months later, a new contrast-enhanced abdominal CT scan was repeated and revealed apart from the known masses an increase in the size of the tumor in the left lobe of the liver (diameter from 4 cm to 7 cm). On unenhanced scans all masses appeared well-defined and homogeneously hypodense. Following an i.v. bolus of contrast, the masses showed early arterial contrast enhancement, with the exception of a centrally located area in the largest lesion, which remained hypodense (Figure 2A and B). On portal venous phase scans all lesions became isodense. The central hypodense area of the largest lesion remained unchanged during the portal venous phase scans, while became hyperdense on delayed scans. This area was attributed to a central scar, a finding consistent with a typical FNH. The remaining three masses were also con-

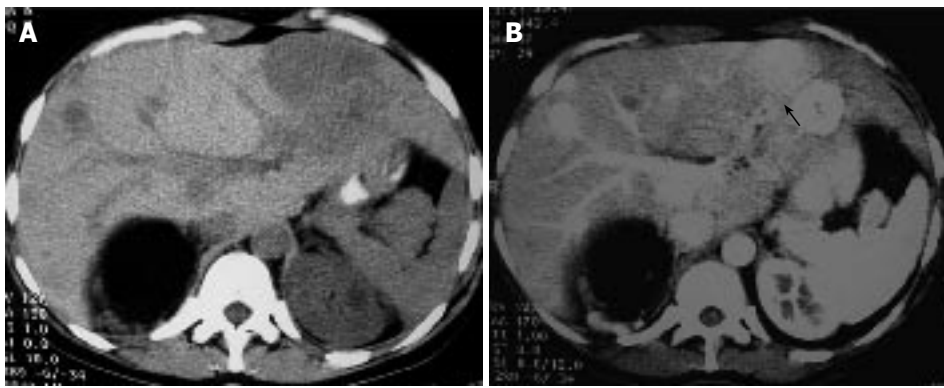


Figure 1 **A:** Unenhanced transverse CT scan demonstrates multiple hypodense masses located on both liver lobes. Fatty tissue (known angiomyolipoma) replaces the most of the upper pole of the right kidney; **B:** Post-contrast CT scan depicts multiple round liver lesions, with a smooth margin, which demonstrate intense homogeneous enhancement. The lesion located in segment three, has a small central area of hypodensity, consistent with a central scar.

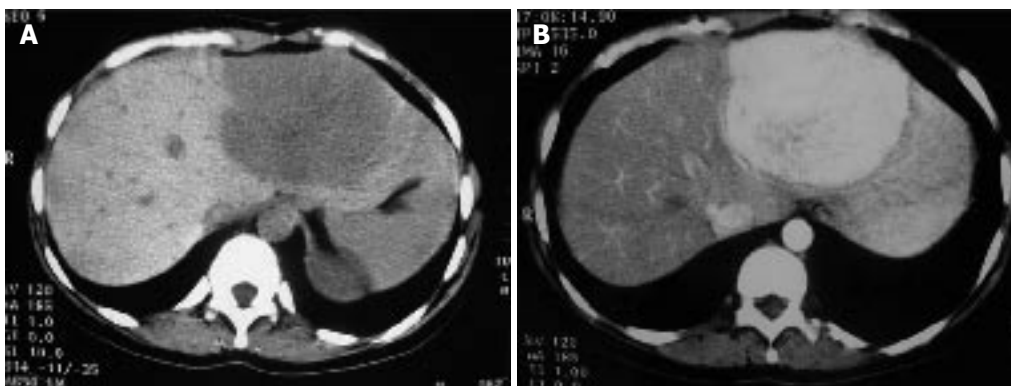


Figure 2 **A:** Pre-contrast CT scan 3 mo later, revealing the significant increase in the size of the lesion located in the left liver lobe; **B:** Post-contrast image at the same level showing the increased contrast uptake of the lesion.

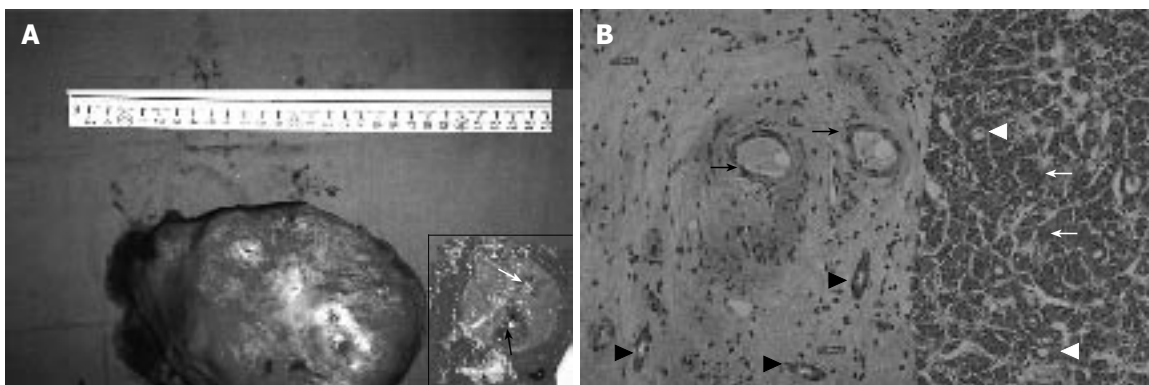


Figure 3 **A:** Photograph of the surgical specimen. The inset shows the cut surface: White arrow points at the scar of FNH and black arrow shows the hepatocellular carcinoma; **B:** Photomicrograph from the lesion. Left: Connective tissue from the core of FNH containing thin wall vessels (black arrows) and cholangioles (black arrowheads). Right: hepatocellular carcinoma. The tumor shows a trabecular growth pattern (white arrows) and focal pseudoglandular transformation (white arrowheads) (HE x 100).

sidered as FNHs. MR imaging was carried out at the same time and the findings were consistent with multiple FNHs.

Due to the great increase in the size of the mass located in the left lobe, during such a short period (3 mo), a CT guided core liver biopsy was performed in the largest lesion. Pathologic examination of the biopsy revealed that the specimen composed of fibrous tissue surrounded a nodule of hyperplastic hepatocytes, contained numerous thin-walled vessels, as well as numerous proliferated bile ductules. No evidence of malignancy was observed. Based on the radiological and histopathological data, the provisional diagnosis of FNH was made. In order to exclude any coexistent brain pathology, the patient underwent brain MR imaging, which was normal.

The surgical procedure involved the resection of the

II and III liver segments. During the operation a frozen section was performed, revealing FNH. The surgical specimen measured 17.3 cm × 15.0 cm × 10.2 cm (Figure 3A). It consisted of a portion of liver which contained two masses, the larger measuring 9 cm × 6 cm × 5 cm and the smaller 4.5 cm × 3.5 cm × 2.0 cm. Macroscopically, both masses had a yellow-white cut surface and a central scar. In addition, the smaller mass contained another smaller tumor measuring 2.1 cm × 1.8 cm × 1.0 cm, which was located in the periphery and showed a brown-green cut surface (Figure 3A-inset). Microscopic examination of the specimen revealed the presence of FNH (two discrete tumors), whereas the smaller tumor included within the smaller mass represented a well-differentiated hepatocellular carcinoma (Figure 3B). Diagnosis of HCC was based

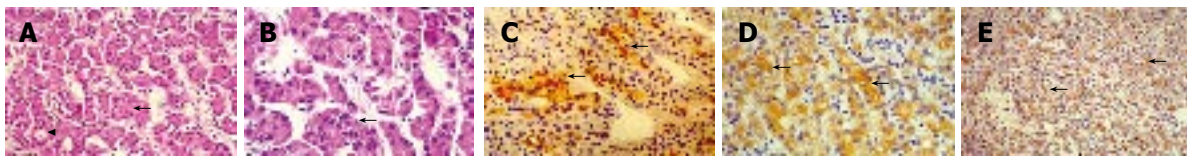


Figure 4 Microphotograph showing details of the tumor. **A, B:** The tumor displays a trabecular growth pattern (**A**-arrow, **B**-arrow) with focal pseudoglandular transformation (**A**-arrowhead) (HE, **A** × 250, **B** × 400); **C:** Tumor cells show positive immunohistochemical stain for hepatocyte antigen (arrows-streptavidin biotin peroxidase × 250); **D:** Tumor cells show positive immunohistochemical stain for cytokeratin 18 (arrows-streptavidin biotin peroxidase × 400); **E:** Immunohistochemical stain for polyclonal carcinoembryonic antigen displays a canalicular pattern (arrows-streptavidin biotin peroxidase × 100).

on morphological (HE stain) and immunohistochemical grounds (Figure 4): The neoplastic cells exhibited positive stain for hepatocyte paraffin antigen and cytokeratin 18 and negative stain for cytokeratin CK19, chromogranin, synaptophysin, common leukocyte antigen negative, CD34 antigen. In addition, polyclonal carcinoembryonic antigen displayed a canalicular pattern.

The postoperative period was uneventful. During the follow-up period, the patient underwent liver transplantation in another center abroad. Pathologic examination at that time revealed that the two small tumors in the right liver lobe represented liver angiomyolipomas (personal communication). At present time, eight years after the initial diagnosis, the patient is alive.

DISCUSSION

Benign liver tumors are uncommon in surgical practice (3%-5% of all liver tumors); haemangioma is the most common type (55%-60%), whereas adenomas (8%-19%) and FNH (21%-27%) are less frequent. FNH, also called mixed tumor represents a small percentage (1%-5%); its definition is derived from the coexistence of two or more tumor phenotypes^[1,12,13]. FNH is generally considered to be a hyperplastic response to an abnormal blood supply^[14].

The potential of benign liver tumors for malignant degeneration has been extensively discussed in the literature; in particular, a considerable risk of malignant transformation has been reported for hepatic adenoma^[15], which was supported by findings of HCC foci within the benign tumor mass. This is why surgical resection of hepatic adenomas is recommended by most authors (apart from considerations about possible rupture of larger adenomas and subsequent intra-abdominal bleeding).

Conversely, there is no agreement on the malignant potential of FNH. To the best of our knowledge only a few cases of co-existent FNH and HCC have been reported in the literature^[8,11] and the pathological correlation is not always clear. Saul *et al* reported one case in 1987 in which FNH and HCC were concomitant, although the HCC was a fibrolamellar variant (FL-HCC)^[9]; Chen *et al* recently reported one case of HCC partially surrounded by FNH^[8], while even more recently Cucchetti *et al* reported a case of simultaneous presence of FNH and HCC^[11].

Simultaneous appearance of FNH with adenoma^[16,17] and haemangioma^[18] has been reported. The simultaneous occurrence of adenoma, focal nodular hyperplasia and haemangioma has also been described and the authors have

concluded that adenoma, FNH and haemangiomas might have a common origin that should be attributed to vascular changes primarily caused by a congenital abnormality of the angioarchitecture and blood circulation of the liver^[19]. Various authors have described the co-existence of FNH with vascular cranial malformations^[20], cystic dysplasia of the kidneys^[21], Klippel-Trenaunay syndrome^[22]. In all cases described so far, the coexistence of the above findings were attributed to a so called syndrome or were described as coincidental^[23]. A useful diagnostic tool for the distinction between liver cell adenoma and FNH, when the routine histopathologic features are not very clear, is clonality analysis^[24].

The histopathological features of FL-HCC suggest a direct link between this tumor and FNH and some authors hypothesize a direct evolution from FNH to FL-HCC^[9,10]. Also, the simultaneous presence of adjacent adenoma does not exclude the development of HCC from malignant degeneration of the adenoma. Therefore co-existence of FNH and HCC in the same patient is an exceptional event, to the best of our knowledge reported only in two cases.

Recent insight into the molecular characteristics of the clonal growth of FNH failed to support further a possible derivation of HCC from FNH; two different studies, conducted by Gaffey^[12] and Paradis^[13], were undertaken to clarify the monoclonal or polyclonal nature of these tumors by a method that scans the molecular pattern of inactivation of chromosome X. The authors eventually came to opposite conclusions. In another study^[8], clonal analysis was applied to macroscopically different portions having different histological features within the same tumor, with concomitant FNH and HCC; the results showed that FNH was of monoclonal origin, but the FNH clone was similar to that of HCC and therefore the authors' conclusions did not support the hypothesis that HCC was the product of malignant transformation from FNH. The issue of an identical clonal origin of FNH and HCC therefore remains a matter of debate, although we feel it is rather imprudent at present to completely exclude any malignant potential of FNH.

The potential of FNH for malignant evolution would appear unlikely on the basis of the follow-up of patients with non-surgically excised FNH. Weimann *et al* observed an increase in the size of FNH lesions only in 9.5% of 53 cases, with no malignant transformation during a mean follow-up of three years^[25]. Likewise, in other studies no increase in lesion size was observed among 11 patients in a two-year follow-up, and in some cases the FNH even completely disappeared over time^[26]. This evidence

recently led Charny *et al*, to conclude that, if the diagnosis is unequivocal, surgical resection is not indicated for asymptomatic FNH^[27].

A clear and precise diagnosis of a benign liver tumor is difficult to obtain in every patient, particularly in the case of FNH. In the series collected by Terkivatan, difficulty in differentiating FNH from adenoma or HCC represented the indication for surgery in 32% of cases^[28]. FNH was rarely encountered by radiologists prior to the current practice of helical multiphasic CT or MR imaging. It is also believed that as the imaging methods improve, FNH will be encountered more frequently. Many authors have reported the CT features of FNH. Imaging characteristics, typical of FNH, include a homogeneous well-defined hypo-, or iso-dense lesion on unenhanced scans, which shows bright enhancement during early arterial phase and becomes isodense on portal venous phase. Central scars are hypodense on early arterial and portal venous phases and become hyperdense on delayed scans^[29-32]. Magnetic resonance imaging is another modality also used for the confident diagnosis of FNH. The reported sensitivity and specificity values for contrast enhanced-MRI diagnosis of FNH was 70% and 98% respectively in an article by Cherqui *et al* in a series of 41 patients with clinical radiological and pathological correlations. The central scar was detected in 78% of the cases^[33]. An alternative to angiography could be hepatic cholescintigraphy, which according to the study of Weimann, best reflected the vascular pattern and the typical biliary ductule proliferation of FNH (sensitivity $\geq 82\%$, specificity $\geq 97\%$)^[25].

Finally, the diagnostic value of liver biopsy in suspected FNH is rather limited; the lesion itself may not be reached or the specimen may not be sufficient for an accurate diagnosis, and false negative results are deleterious for the final outcome as hepatic adenomas or HCC may bleed or seed along the needle track.

In conclusion, at present there is no clear-cut evidence supporting the potential for malignant degeneration of FNH into HCC; the indication for surgery, particularly in small lesions (≤ 4 cm) and asymptomatic patients, is therefore rather controversial. In view of this uncertainty, a correct diagnosis which differentiates between FNH and HCC must be achieved for all cases by means of a multidisciplinary approach. Due to the rarity of the association between FNH and HCC it is difficult to draw solid conclusions for both the pathology of this entity and the appropriate management of these patients.

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20-25 October 2006
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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
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Endoscopy
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3-7 June 2006
Seattle - Washington
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10th World Congress of the International
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22-25 February 2006
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Inflammatory Bowel Diseases
24-25 March 2006
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Falk Foundation e.V.
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10th International Congress of Obesity
3-8 September 2006
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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
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International Gastrointestinal Fellows
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www.cag-acg.org

Canadian Digestive Disease Week
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Digestive Disease Week Administration
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www.cag-acg.org

Prague Hepatology 2006
14-16 September 2006
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Foundation of the Czech Society of
Hepatology
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Congresscare
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www.colorectal2006.org

Falk Symposium 152: Intestinal Disease
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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 Xiaohua Zazhi* 1999; **7**: 285-287

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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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- 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]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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- 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]

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- 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]

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- 9 Outreach: bringing HIV-positive individuals into care. *HRS/A 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

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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

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>

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- 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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